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ORIGINAL ARTICLE

Direct one-step labeling of cysteine residues on peptides with [^{11}C]methyl triflate for the synthesis of PET radiopharmaceuticals

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Abstract Radiolabeled peptides have emerged as an attractive platform for the diagnostic and therapeutic oncology. However, the ^{11}C-radiolabeling of peptides for positron emission tomography (PET) has been poorly explored, owing to the relatively short half-life of carbon-11 ($t_{1/2} = 20.3$ min) and time-consuming multi-step radiochemical reactions. Existing methods have found limited use and are not routinely encountered in the production of radiotracers. Herein, we propose a facile one-step direct ^{11}C-methylation of cysteine residues in peptides using [^{11}C]methyl triflate under ambient temperatures (20 °C) and short reaction times, on the order of seconds. Good regioselectivity of this method was demonstrated by HPLC in a simple peptide (glutathione, GSH) and a more complex test decapeptide (Trp-Tyr-Trp-Ser-Arg-Cys-Lys-Trp-Thr-Gly) bearing multiple nucleophilic sites. In addition, we extend this method towards the synthesis of [^{11}C]Cys(Me)-[Tyr^3-octreotate] as a demonstration of applicability for peptides of biological interest. This octreotate derivative was obtained in non-decay-corrected radiochemical yields of 11 ± 2 % ($n = 3$) with a synthesis time of approx. 30 min.

Keywords Solid-phase peptide synthesis · Tyr^3-octreotate · Positron emission tomography · Carbon-11 · [^{11}C]methyl triflate · Radiochemistry

Abbreviations

PET	Positron emission tomography
GSH	Glutathione
SPPS	Solid-phase peptide synthesis
SST	Somatostatin
SPECT	Single-photon emission computed tomography
[^{11}C]MB-CHO	*p*-[^{11}C]methyoxybenzaldehyde
HPLC	High-performance liquid chromatography
[^{11}C]MeOTf	[^{11}C]methyl trifluoromethylsulfonate; [^{11}C]methyl triflate
Gly-Sar	Glycylsarcosine
DMS	Dimethyl sulfate
MMS	Methyl methanesulfonate
CNSC	Canadian Nuclear Safety Commission
Fmoc	Fluorenylmethyloxycarbonyl
HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate

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DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
TFA	Trifluoroacetate; trifluoroacetic acid
TIPS	Triisopropylsilane
HRMS	High-resolution mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization/time-of-flight
DMSO	Dimethyl sulfoxide
GSMe	*S*-methylglutathione
RP-HPLC	Reversed-phase high-performance liquid chromatography
MEK	Methyl ethyl ketone
MeCN	Acetonitrile
ndc	Non-decay-corrected
RCY	Radiochemical yield
EtOH	Ethanol

Introduction

Radiolabeled peptides have emerged as an attractive platform for the diagnostic and therapeutic oncology. Advances in solid-phase peptide synthesis (SPPS) and well-established bioconjugation techniques have allowed for the ease of preparation, chemical modification, and radiolabeling of these compounds (Okarvi 2004; Ambrosini et al. 2011). In particular, small peptides (8-20 amino acids) show promise in nuclear imaging as they exhibit rapid pharmacokinetics, excellent tissue permeability, reduced immunogenicity, and good tumor targeting characteristics due to overexpressed peptide receptors in many cancers (Weiner and Thakur 2001; Reubi 2003). To this date, ^{111}In-DTPA^{0}-octreotide (^{111}In-OctreoScan, ^{111}In-pentetreotide) remains the first and most successful radiotracer for imaging somatostatin (SST) receptor-positive lesions in single-photon emission computed tomography (SPECT) (Rufini et al. 2006; Fani et al. 2012). Due to inherently superior detection sensitivity and temporal resolution of the positron emission tomography (PET) modality (Rahmim and Zaidi 2008), various SST analogs labeled with positron-emitting copper-64 [^{64}Cu-CB-TE2A-Y^{3}-TATE (Sprague et al. 2004)], fluorine-18 [Gluc/Cel-S-Dpr([^{18}F]FBOA)TOCA (Schottelius et al. 2004), Glu-Lys([^{18}F]FP)-TOCA (Meisetschläger et al. 2006), [^{18}F]FP-Glu-TOCA (Wester et al. 2003), SiFA-Asn(AcNH-β-Glc)-PEG-Tyr^{3}-octreotate (Wängler et al. 2010, 2012), and ^{18}F-AlF-NOTA-OC (Laverman et al. 2010)], and gallium-68 [^{68}Ga-DOTA-TOC (Koukouraki et al. 2006) and ^{68}Ga-DOTA-NOC (Wild et al. 2005)] have been developed and are under preclinical or clinical evaluation.

Carbon-11 remains poorly explored in peptide labeling, owing to its relatively short half-life ($t_{1/2}$ = 20.3 min) and time-consuming, multi-step radiochemical reactions generally associated with these complex biomolecules. The earliest reported synthesis of ^{11}C-labeled peptides was the labeling of methionine residues, specifically the tandem reductive deprotection and subsequent [^{11}C]methyl iodide ([^{11}C]CH_3I) methylation of pentameric enkephalin peptides bearing benzyl-protected homocysteine residues (Långström et al. 1981; Hartvig et al. 1986), and further adopted for the synthesis of neuropeptide [^{11}C]substance P (RPKPQQFFGL[^{11}C]M) (Franzén et al. 1987). However, this technique failed to see widespread adoption due to reaction conditions difficult to implement for routine production of radiotracers (Lundqvist and Tolmachev 2002). Recently, *p*-[^{11}C]methyoxybenzaldehyde ([^{11}C]MB-CHO) derived from [^{11}C]CH_3I has been developed as a prosthetic group for the chemoselective labeling of an aminooxyacetic acid-functionalized analog of Tyr^{3}-octreotate (Henriksen et al. 2004). However, this two-step labeling proceeds in an optimal synthesis time of 1 h, owing to the need for two radio-high-performance liquid chromatography (HPLC) purifications. Labeling using [^{11}C]methyl trifluoromethylsulfonate ([^{11}C]methyl triflate, [^{11}C]MeOTf) has been reported with the dipeptide glycylglycine ethyl ester on the amino-terminus as the first step towards the radiosynthesis of [^{11}C]Glycylsarcosine (Gly-Sar) as PET tracer of peptide transporter PepT2 (Nabulsi et al. 2005).

The direct, nonradioactive methylation of peptides and proteins has been previously reported using methyl sulfonates with high regioselectivity. Methyl *p*-nitrobenzenesulfonate (methyl nosylate) was used to selectively methylate the histidine-57 residue of α-chymotrypsin (Nakagawa and Bender 1969) and cysteine residues of egg white lysozyme and insulin B chain in the presence of lysine, histidine, methionine, tyrosine, and serine residues (Heinrikson 1970). Treatment of nuclear proteins with dimethyl sulfate (DMS) or methyl methanesulfonate (MMS) shows methylation at cysteine and histidine residues (Boffa and Bolognesi 1985). The methylation of metal-bound cysteinates on zinc finger peptides by DMS has also been reported (Roehm and Berg 1998).

Herein we propose a facile, one-step direct ^{11}C-methylation of cysteine residues in peptides using [^{11}C]MeOTf. Specifically, we apply this method towards the labeling of a simple, commercially available peptide (glutathione, GSH) and a more complex decapeptide (Trp-Tyr-Trp-Ser-Arg-Cys-Lys-Trp-Thr-Gly) bearing multiple nucleophilic sites to demonstrate regioselectivity of radiomethylation. Furthermore, we extend this method towards the synthesis of [^{11}C]Cys(Me)-[Tyr^{3}-octreotate] ([^{11}C]**10**) as a demonstration of applicability towards peptides of biological interest.

Materials and methods

Regulatory notice

All manipulations involving radioactive materials have been performed in full accordance with Canadian Nuclear Safety Commission (CNSC) regulations.

Materials

Unless stated otherwise, commercially available chemical compounds were purchased from Sigma-Aldrich (Oakville, ON, Canada) in the highest purity and used without further purification. Fmoc-protected resins and amino acids were purchased from NovaBiochem (Merck KGaA, Darmstadt, Germany) with the exception of Fmoc-Cys(Me)-OH, which was purchased from Bachem (Torrance, CA, USA), Fmoc-Arg(Pbf)-OH, which was purchased from GL Biochem (Shanghai, China), and Fmoc-Ser(Me)-OH and Fmoc-Thr(Me)-OH, which were purchased from Iris Biotech (Marktredwitz, Germany). Bulk solvents were rated HPLC grade or higher, and purchased from Fischer Scientific (Waltham, MA, USA). [^{11}C]CO_2 was prepared at the cyclotron unit of the McConnell Brain Imaging Centre, Montreal Neurological Institute. SepPak QMA and C-18 light cartridges were obtained from Waters Corporation (Milford, MA, USA).

Instruments

Semi-preparative HPLC was performed on an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA; running on Agilent ChemStation software) equipped with a MZ semi-prep LiChrosorb column (RP-Select B 5 µm, 250 × 10 mm; Merck, Germany) at a flow rate of 2 mL/min, and UV detection was performed at 230 and 254 nm. Analytical HPLC was performed on an Agilent 1200 system equipped with a Raytest Gabi Star radioactivity detector (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany) and either a Chromolith Performance column (RP-18e, 100 × 4.6 mm; Merck, Germany) at a flow rate of 4.0 mL/min or an Aeris PEPTIDE column (XB-C18 3.6 µm, 250 × 4.6 mm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.8 mL/min, and UV detection was performed at 210, 230, and 254 nm. HPLC mobile phases consist of eluent A (0.1 % TFA in MeCN) and eluent B (0.1 % TFA in H_2O) filtered through a 0.45-µm nylon membrane filter and degassed before use. Different HPLC methods were used depending on choice of peptide and column (Table 1). All samples for preparative and analytical HPLC were diluted with a minimum of 50 % v/v eluent B prior to injection. Samples were injected via a model 7725i Rheodyne syringe loading sample injector fitted with a 2-mL loop (Rheodyne, Cotati, CA, USA). Gamma counting was performed using a CRC-25PET Dose Calibrator (Capintec, Ramsey, NJ, USA). Mass spectrometry of peptides was performed using a Brucker Microflex LT MALDI-TOF MS (Brucker Daltonics, Billerica, MA, USA).

Peptide synthesis: general procedure for the synthesis of **1–12**

Peptides **1–12** (Fig. 1) were synthesized on solid support using standard Fmoc SPPS as described by Wellings and Atherton (1997) on a Fmoc-Thr(tBu)-Wang or a Fmoc-Gly-Wang resin [standard coupling: 4 equiv. amino acid, 4 equiv. HBTU, 4 equiv. DIPEA in DMF; Fmoc deprotection: 50 % piperidine in DMF; disulfide cyclization: 4 equiv. Tl(TFA)$_3$ in DMF]. The peptides were cleaved from the resin and deprotected upon treatment with a solution of TFA/TIPS/H_2O (95:2.5:2.5, 2 mL) for 60 min, and then precipitated from solution upon the addition of excess diethyl ether. The crude solid was washed three times with excess diethyl ether, and purified by semi-preparative HPLC (**1–8**: Method A; **9–12**: Method B) to afford the desired peptide as a white solid after lyophilization with a purity of 95 % or greater determined by quality control (HPLC Method E).

Synthesis of Trp-Tyr-Trp-Ser-Arg-Cys-Lys-Trp-Thr-Gly (**1**) 32 % yield. $R_{t(\text{Method A})} = 7.6$ min. HRMS (MALDI-TOF, *m/z*) for $C_{66}H_{86}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,372.55 (1,372.63).

Synthesis of Trp-Tyr-Trp-Ser-Arg-Cys(Me)-Lys-Trp-Thr-Gly (**2**) 48 % yield. $R_{t(\text{Method A})} = 7.7$ min. $R_{t(\text{Method E})} = 18.9$ min. HRMS (MALDI-TOF, *m/z*) for $C_{67}H_{88}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,386.71 (1,386.64).

Synthesis of Trp-Tyr-Trp-Ser-Arg-Cys-Lys(Me)-Trp-Thr-Gly (**3**) 16 % yield. $R_{t(\text{Method A})} = 7.6$ min. $R_{t(\text{Method E})} = 18.4$ min. HRMS (MALDI-TOF, *m/z*) for $C_{67}H_{88}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,386.68 (1,386.64).

Synthesis of Trp-Tyr-Trp-Ser-Arg(Me)-Cys-Lys-Trp-Thr-Gly (**4**) 6 % yield. $R_{t(\text{Method A})} = 7.8$ min. $R_{t(\text{Method E})} = 18.4$ min. HRMS (MALDI-TOF, *m/z*) for $C_{67}H_{88}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,386.81 (1,386.64).

Synthesis of Trp-Tyr(Me)-Trp-Ser-Arg-Cys-Lys-Trp-Thr-Gly (**5**) 44 % yield. $R_{t(\text{Method A})} = 7.7$ min. $R_{t(\text{Method E})} = 22.7$ min. HRMS (MALDI-TOF, *m/z*) for $C_{67}H_{88}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,386.74 (1,386.64).

Synthesis of Trp(α-N-Me)-Tyr-Trp-Ser-Arg-Cys-Lys-Trp-Thr-Gly (**6**) 51 % yield. $R_{t(\text{Method A})} = 7.6$ min. $R_{t(\text{Method E})} = 17.9$ min. HRMS (MALDI-TOF, *m/z*) for $C_{67}H_{88}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,386.68 (1,386.64).

Synthesis of Trp-Tyr-Trp-Ser-Arg-Cys-Lys-Trp-Thr(Me)-Gly (**7**) 33 % yield. $R_{t(\text{Method A})} = 7.8$ min. $R_{t(\text{Method E})}$

Table 1 List of analytical and preparative HPLC elution methods used in the current study

Method	Column	Flow (mL/min)	Gradient timeline (min)	Eluent A (%)	Eluent B (%)	Elution
A	LiChrosorb	2.0	0.00	25	75	Linear gradient
			40.00	100	0	Linear gradient
			45.00	25	75	Equilibration
B	LiChrosorb	2.0	0.00	0	100	Linear gradient
			30.00	100	0	Linear gradient
			35.00	0	100	Equilibration
C	Aeris PEPTIDE	0.8	0.00	0	100	Linear gradient
			5.00	15	85	Linear gradient
			15.00	20	80	Linear gradient
			17.50	100	0	Linear gradient
			20.00	100	0	Isocratic
			25.00	0	100	Equilibration
D	Chromolith Performance	4.0	0.00	0	100	Linear gradient
			10.00	100	0	Linear gradient
			12.00	0	100	Equilibration
E	Aeris PEPTIDE	0.8	0.00	0	100	Linear gradient
			5.00	25	75	Linear gradient
			15.00	35	65	Linear gradient
			17.50	100	0	Linear gradient
			20.00	100	0	Isocratic
			25.00	0	100	Equilibration

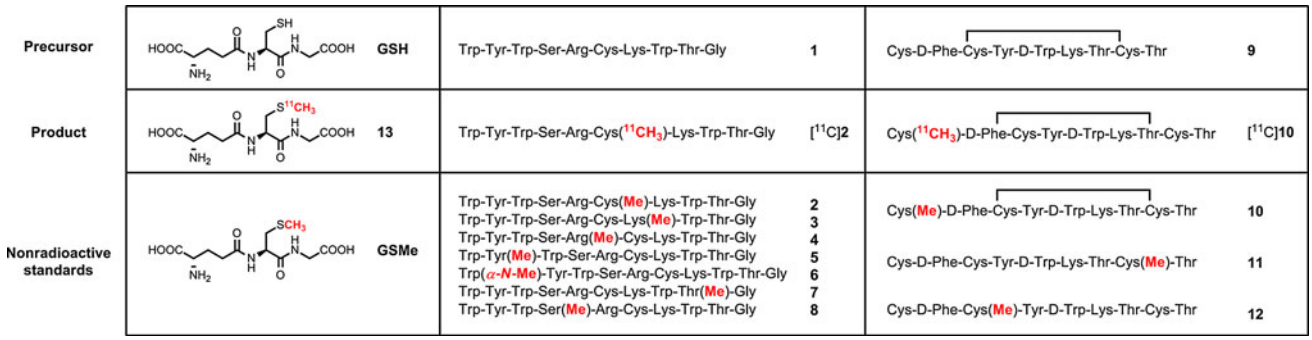

Fig. 1 List of peptidic precursors, radiolabeled products, and nonradioactive standards employed in this study

= 19.9 min. HRMS (MALDI-TOF, *m/z*) for $C_{67}H_{88}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,386.47 (1,386.64).

Synthesis of Trp-Tyr-Trp-Ser(Me)-Arg-Cys-Lys-Trp-Thr-Gly (**8**) 26 % yield. $R_{t(Method\ A)}$ = 7.7 min. $R_{t(Method\ E)}$ = 19.3 min. HRMS (MALDI-TOF, *m/z*) for $C_{67}H_{88}N_{17}O_{14}S^+$ $[M + H]^+$ (calc'd): 1,386.30 (1,386.64).

Synthesis of Cys-[Tyr^3-octreotate] (**9**) 23 % yield. $R_{t(Method\ B)}$ = 17.7 min. HRMS (MALDI-TOF, *m/z*) for $C_{52}H_{70}N_{11}O_{13}S_3^+$ $[M + H]^+$ (calc'd): 1,152.46 (1,152.43).

Synthesis of Cys(Me)-[Tyr^3-octreotate] (**10**) 14 % yield. $R_{t(Method\ B)}$ = 17.9 min. $R_{t(Method\ E)}$ = 18.8 min. HRMS (MALDI-TOF, *m/z*) for $C_{53}H_{72}N_{11}O_{13}S_3^+$ $[M + H]^+$ (calc'd): 1,166.37 (1,166.45).

Synthesis of Cys-[Cys(Me)^2, Tyr^3, Cys(SH)^7-octreotate] (**11**) 18 % yield. $R_{t(Method\ B)}$ = 18.3 min. $R_{t(Method\ E)}$ = 20.7 min. HRMS (MALDI-TOF, *m/z*) for $C_{53}H_{74}N_{11}O_{13}S_3^+$ $[M + H]^+$ (calc'd): 1,168.47 (1,168.46).

Synthesis of Cys-[Cys(SH)^2,Tyr^3,Cys(Me)^7-octreotate] (**12**) 16 % yield. $R_{t(Method\ B)}$ = 18.1 min. $R_{t(Method\ E)}$ = 20.2 min. HRMS (MALDI-TOF, *m/z*) for $C_{53}H_{74}N_{11}O_{13}S_3^+$ $[M + H]^+$ (calc'd): 1,168.58 (1,168.46).

Production of [^{11}C]methyl iodide

Carbon-11 was produced in the form of $[^{11}C]CO_2$ from a Cyclone® 18/9 cyclotron (IBA, Louvain-La-Neuve, Belgium) by irradiation of N_2 containing 0.5 % O_2 using the

$^{14}N(p,\alpha)^{11}C$ nuclear reaction with 18 MeV protons. [^{11}C]CH_3I was prepared by an automatic synthesis module (MeI Module, Scintomics, Fürstenfeldbruck, Germany) via a series of reactions previously described by Marazano et al. (1977). In brief, [^{11}C]CO_2 was reduced via $LiAlH_4$ and subsequently converted to [^{11}C]CH_3I by treatment with hydroiodic acid under reflux conditions.

Production of [^{11}C]methyl triflate

[^{11}C]MeOTf was synthesized as previously reported by Jewett (1992). In brief, [^{11}C]MeOTf was generated by reaction of freshly produced [^{11}C]CH_3I with 50 mg of silver triflate (50 mg graphitized carbon solid support; glass column) in an online flow-through process at 175 °C using a N_2 gas flow of 20 mL/min. The freshly produced [^{11}C]MeOTf in N_2 carrier gas was used immediately for subsequent radiomethylation reactions.

General procedure for the radiosynthesis of [^{11}C]GSMe (**13**)

To a freshly prepared solution of glutathione (300 µg, 0.98 µmol) and aqueous NaOH (9 µL, 0–4.0 M, 0–24 µmol, 0–24 eq) in DMSO (300 µL) was bubbled freshly produced [^{11}C]MeOTf (185 MBq–1.11 GBq) in N_2 carrier gas at room temperature (20 °C) for 20 s without stirring. The reaction was subsequently quenched with TFA (4.5 µL, 59 µmol) and analyzed by reverse-phase HPLC (Method C) for radiochemical yield determination.

General procedure for the radiosynthesis of Trp-Tyr-Trp-Ser-Arg-Cys($^{11}CH_3$)-Lys-Trp-Thr-Gly ([^{11}C]**2**)

To a freshly prepared solution of **1** (300 µg, 0.22 µmol) and aqueous NaOH (2.1 µL, 0–4.0 M, 0–48 eq) in DMSO (300 µL) was bubbled freshly produced [^{11}C]MeOTf (185 MBq–1.11 GBq) in N_2 carrier gas at room temperature (20 °C) for 20 s without stirring. The reaction was subsequently quenched with TFA (4.5 µL, 59 µmol) and analyzed by reverse-phase HPLC (Method D or E) for radiochemical yield determination and regioselectivity of methylation.

General procedure for the radiosythesis of Cys($^{11}CH_3$)-[Tyr^3-octreotate] ([^{11}C]**10**)

To a freshly prepared solution of **9** (500 µg, 0.43 µmol) and aqueous NaOH (7.2 µL, 2.0 M, 40 eq) in DMSO (300 µL) was bubbled freshly produced [^{11}C]MeOTf (370 MBq – 4.81 GBq) in N_2 carrier gas at room temperature (20 °C) for 20 s without stirring. The reaction was subsequently quenched with TFA (4.5 µL, 59 µmol) and analyzed and/or purified by reverse-phase HPLC (Method D or E).

Preparative isolation of [^{11}C]**10**

The appropriate reverse-phase HPLC fraction corresponding to the desired radiolabeled peptide was added to sodium phosphate buffer (8 mL, 0.05 M, pH 7.4) and loaded on a Waters SepPak C-18 light cartridge [preconditioned by subsequent rinsing with ethanol (10 mL) and water (10 mL)]. The trapped radiolabeled peptide was washed with water (5 mL), eluted from the cartridge with ethanol (300–500 µL) and diluted with isotonic saline solution (3–5 mL). The overall synthesis time was 30 min with non-decay-corrected radiochemical yields (RCYs) of 11 ± 2 % ($n = 3$). [^{11}C]**10** was isolated with a maximum radioactivity level of 41.8 MBq and a maximum specific activity of 96.3 MBq/µmol starting from ca. 340 MBq of [^{11}C]MeOTf.

Results and discussion

Radiosynthesis of [^{11}C]GSMe (**13**): effect of solvent and base

In order to evaluate the feasibility of direct one-step ^{11}C-methylation of peptides, preliminary studies were conducted on GSH, a simple, inexpensive peptide whose nonradioactive side-chain methylated standard, *S*-methylglutathione (GSMe), was also available commercially. Radiolabeling was performed using the trifluoromethane sulfonate [^{11}C]MeOTf as methylating agent due to its well-documented route of production (Jewett 1992) and availability as a routine intermediate in most PET cyclotron units for the synthesis of various radiopharmaceuticals. Reversed-phase HPLC (RP-HPLC) method development using an Aeris PEPTIDE column afforded Method C (Table 1) as a satisfactory eluent method for the separation of precursor glutathione (GSH, $R_t = 9.1$ min) and product *S*-methylglutathione (**13**, $R_t = 10.7$ min) (Fig. 2).

Due to the individual setup of the radio-HPLC–UV in this study, there is a significant time delay between the integrated UV and discrete radioactivity detectors. As the identification of radio-HPLC signals is commonly performed via coincident retention time with their respective nonradioactive carbon-12 standards, determination of this time delay is necessary. This flow-dependent time difference (ΔR_t) has been experimentally measured ($\Delta R_t \approx 0.58$ min at 0.8 mL/min; see Supplementary

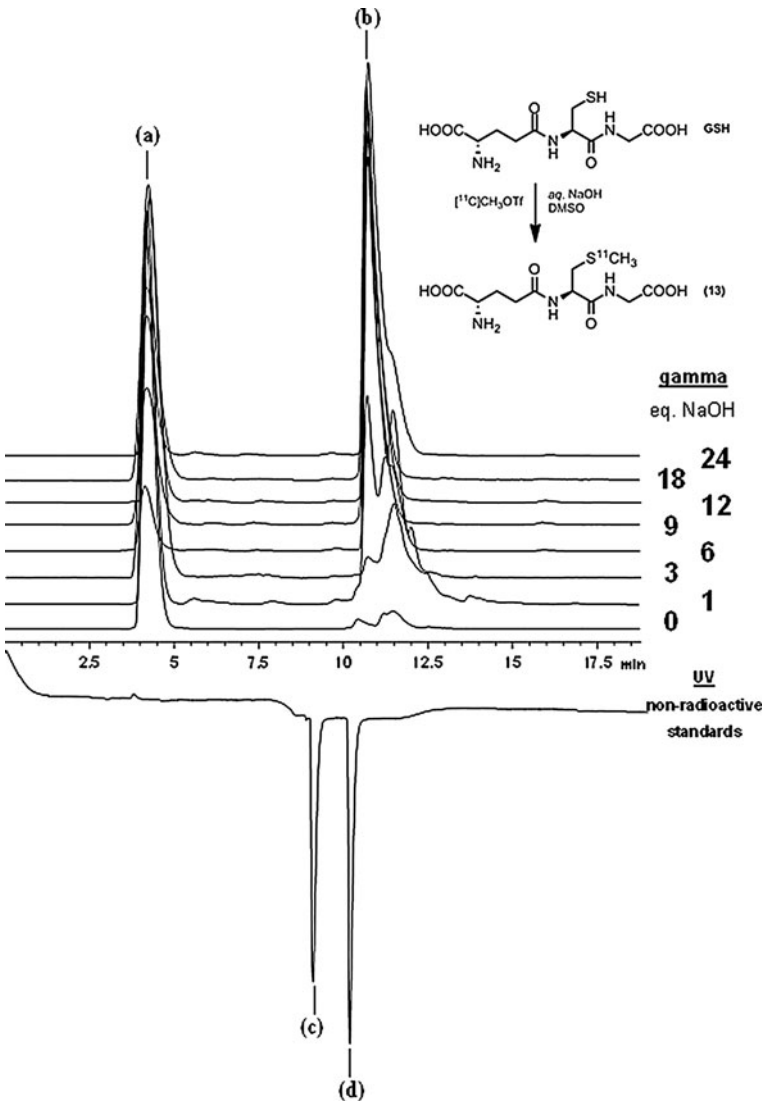

Fig. 2 *Above axis* stacked radio-HPLC chromatograms (Method C) of the radiosynthesis of **13** under varying equivalents of NaOH. Reaction conditions: GSH (300 μg), 2.0 M NaOH (0–24 eq), DMSO (300 μL), [^{11}C]MeOTf (185 MBq–1.11 GBq), rt, 20 s. *Below axis* HPLC chromatogram of GSH and GSMe (Method C). (**a**) [^{11}C]CH_3OH, $R_{t(gamma)} = 4.2$ min. (**b**) **13**, $R_{t(gamma)} = 10.7$ min. (**c**) GSH, $R_{t(210\ nm)} = 9.1$ min. (**d**) GSMe, $R_{t(210\ nm)} = 10.2$ min. Detector delay $\Delta R_t \approx 0.58$ min at 0.8 mL/min

Information) and used throughout this study for the positive identification of radioactive products.

The labeling efficiency of [^{11}C]MeOTf towards GSH was evaluated in the presence of aqueous NaOH using the following three solvents: DMSO, methyl ethyl ketone (MEK), and acetonitrile (MeCN). Care was taken that all DMSO-containing peptide solutions were freshly prepared as DMSO-mediated oxidation of cysteine residues to cystine (disulfide bridge formation) has been documented to occur within hours (Tam et al. 1991). Reaction conditions using 18 equivalents of aqueous NaOH and DMSO as solvent (Fig. 2) afforded appreciable radiochemical conversion of GSH to **13** [65 ± 4.5 % non-decay-corrected (ndc)], and was thereupon used as solvent of choice for optimization of other reaction parameters. Similar labeling conditions using MeCN afforded ca. 23 % ndc radiochemical conversion. Interestingly, MEK, a solvent commonly employed in ^{11}C-radiomethylation reactions, afforded negligible yields of <5 % ndc. Substitution of [^{11}C]MeOTf with [^{11}C]methyl iodide as radiomethylating agent under the above labeling conditions provided no discernible yields of the desired labeled peptide.

When investigating the base-dependence of GSH labeling, at least 6 equivalents of aqueous NaOH were required for appreciable formation (75 % ndc radiochemical conversion) of **13**. This slight excess of base, sufficient for the deprotonation of the two carboxylate and lone thiol moieties in GSH, resulted in a highly nucleophilic thiolate that rapidly reacted with [^{11}C]MeOTf, on the order of seconds to afford **13** as the major radiolabeled product. An excess of up to 24 equivalents of base does not appear to significantly alter the radiochemical yield (RCY) (Fig. 2). Multiple minor peaks that appear in the radiochromatogram under lower equivalents of NaOH were not identified in the present study.

Synthesis of decapeptide **1** and its mono-methylated derivatives **2–8**

In order to determine the regiospecificity of [^{11}C]MeOTf towards cysteine residues on peptides, a more complex decapeptide **1** containing various *N*-, *O*-, and *S*-nucleophilic amino acid side-chains (Fig. 3) was synthesized by standard Fmoc SPPS (Wellings and Atherton 1997). In addition, peptides **2–8** were synthesized as nonradioactive mono-methylated standards for HPLC analysis. These standards, which contain a single methylation group on the side-chain of the following residues: cysteine, lysine, arginine, tyrosine, threonine, and serine along with the *N*-terminal peptide backbone, were chosen as they could be easily synthesized by SPPS standard protocols without additional steps. Following TFA cleavage from solid support, purification via semi-preparative HPLC, and lyophilization, peptides **1–8** were obtained in 6–51 % yields (c.f. "Materials and methods") with a purity of 95 % or greater by quality control (HPLC Method E).

Extensive RP-HPLC method development was required to provide an analytical separation of peptides **2–8** for the assessment of radiomethylation regiospecificity. As these peptides share an identical molecular mass and differ only in the site of attachment of the methyl group, initial efforts in method development using LiChrosorb- or Chromolith-based reversed-phase columns were unable to provide a satisfactory separation of these isomers. Only an Aeris PEPTIDE column (Method E) allowed an adequate separation of anticipated *S*-methylated product **2** from its isomers. Although this method was unable resolve peptides **3** and **4**, the separation of at least 0.5 min between peptide **2** and its isomers (Fig. 4) proved sufficient for use in the subsequent radiosynthesis.

Radiosynthesis of [^{11}C]**2**: regioselectivity and effect of base

Initial investigations into the base-equivalent requirements in the [^{11}C]MeOTf labeling of hexa-protic peptide **1** proved difficult due to instability of this peptide sequence in the basic reaction conditions previously established for GSH labeling. As a result, major unidentified radioactive side-products were observed when pre-treating precursor **1** with any slight excess of NaOH over the course of minutes. In effect, yields of the desired radiopeptide were poor (<20 % ndc) with long reaction times (>1 min) of the radiomethylating agent. Peptide radiomethylation using [^{11}C]MeOTf is a biphasic reaction which involves bubbling a gaseous mixture of [^{11}C]MeOTf in N_2 into the precursor solution. Efforts to minimize the decomposition of **1** and desired product [^{11}C]**2** include the following: (1) use of freshly prepared and degassed peptide precursor solutions, (2) addition of aqueous NaOH in the desired quantities immediately prior to [^{11}C]MeOTf addition, (3) reduction in the time of [^{11}C]MeOTf/N_2 bubbling to 20 s, and (4) acidification via slight excess of TFA at the end of reaction to neutralize NaOH in solution. Efforts to replace aqueous NaOH with solid bases such as cesium carbonate (Cs_2CO_3)

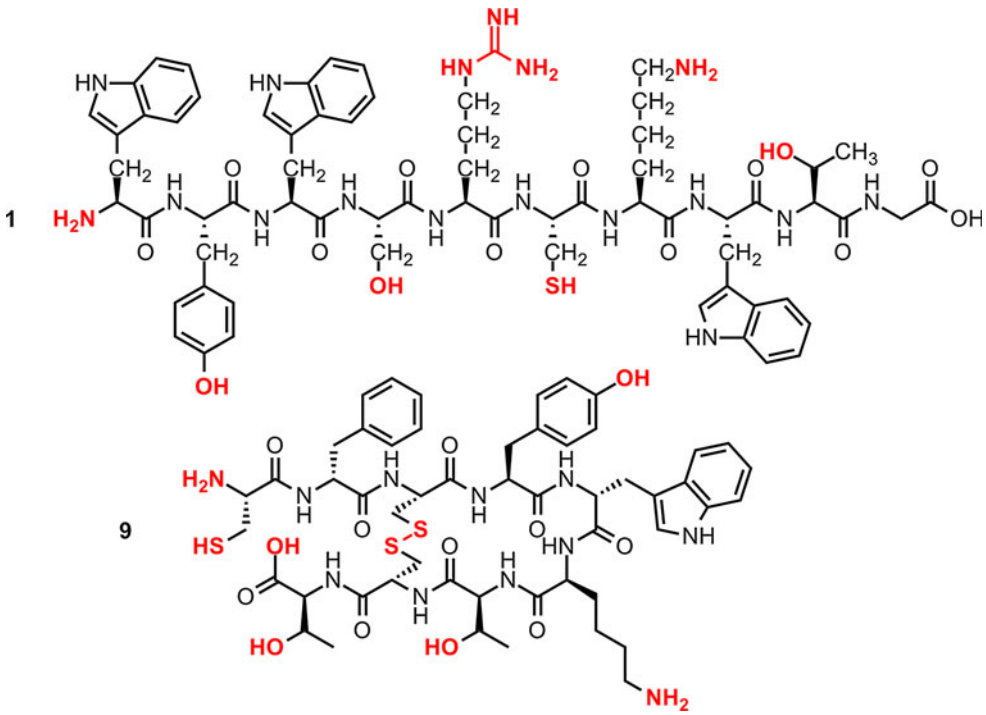

Fig. 3 *Above* reactive sites (*highlighted*) on peptide **1** under investigation and amenable towards radiomethylation under neutral or basic conditions. *Below* potential reactive sites (*highlighted*) on peptide **9** amenable towards radiomethylation under neutral, basic, or reductive conditions

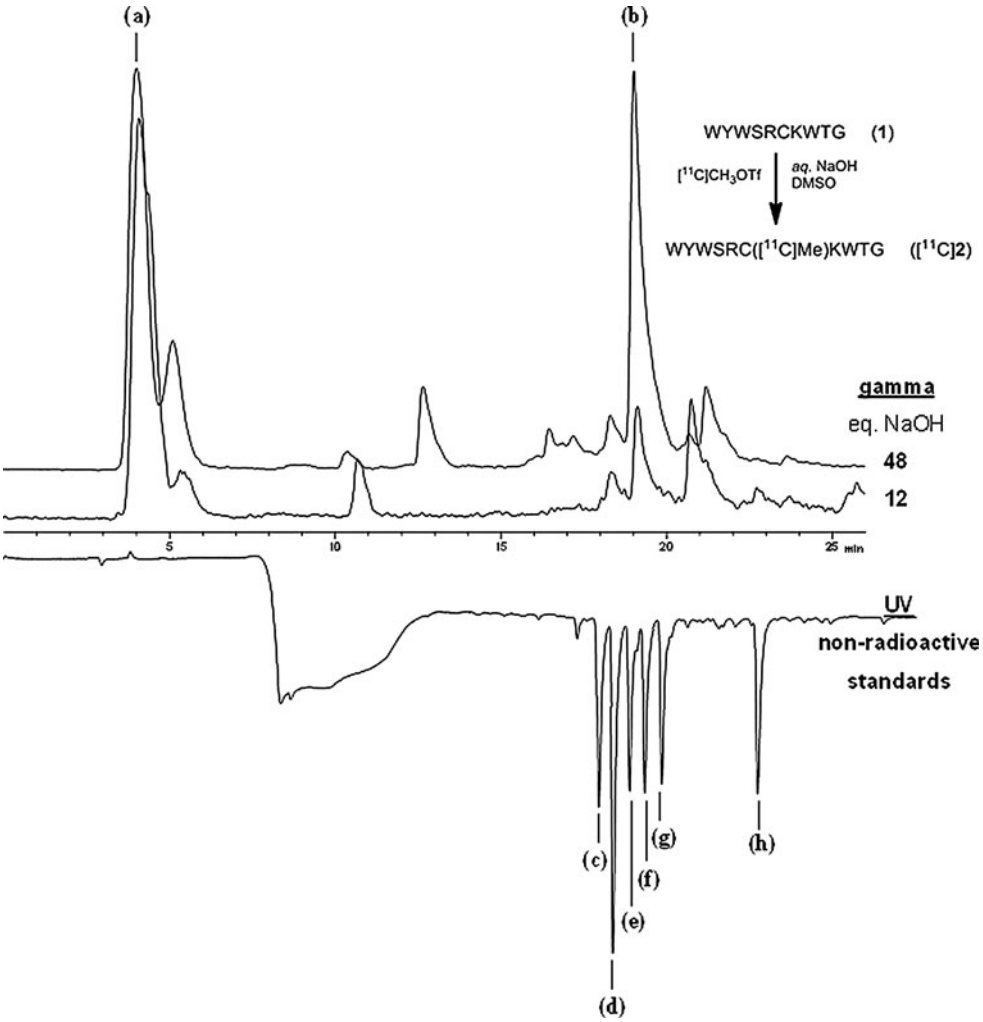

Fig. 4 *Above axis* stacked radio-HPLC chromatograms (Method E) of the radiosynthesis of [^{11}C]**2** under varying equivalents of NaOH. Reaction conditions: **1** (300 μg), 2.0 M NaOH (12 or 48 eq), DMSO (300 μL), [^{11}C]MeOTf (185 MBq–1.11 GBq), rt, 20 s. *Below axis* HPLC chromatogram of **2**–**8** (Method E). (**a**) [^{11}C]CH_3OH, $R_{t(gamma)} = 4.3$ min. (**b**) [^{11}C]**2**, $R_{t(gamma)} = 19.4$ min. (**c**) **6**, $R_{t(210\ nm)} = 17.9$ min. (**d**) Mixture of **3** and **4**, $R_{t(210\ nm)} = 18.4$ min. (**e**) **2**, $R_{t(210\ nm)} = 18.9$ min. (**f**) **8**, $R_{t(210\ nm)} = 19.3$ min. (g) **7**, $R_{t(210\ nm)} = 19.9$ min. (**h**) **5**, $R_{t(210\ nm)} = 22.7$ min. Detector delay $\Delta R_t \approx 0.58$ min at 0.8 mL/min

or freshly powdered NaOH have led to no successful labeling.

TFA was used as choice of acid for quenching excess NaOH in the radiochemical reaction due to its lack of reactivity towards peptide **1**. Following TFA quench, composition of the crude radiomethylation reaction monitored via radio-HPLC remains unchanged over the course of 3 h (see Supplementary Information). The equivalents of acid added was also in lower quantities than reported elsewhere for DMSO/HCl-mediated oxidation of methionine to its sulfoxide (Savige and Fontana 1980), a relevant concern due to a chemical similarity to the expected *S*-[^{11}C]methylcysteine residues of our current study.

Faced with the large volume of planned radiochemical reactions, analytical radio-HPLC acquisitions with long run times of 25 min (Method E), and the short half-life of carbon-11 ($t_{1/2} = 20.3$ min), RP-HPLC method development of a shorter acquisition run was necessary under the high-flow Chromolith Performance column (Method D), albeit at the cost of resolution between radiolabelled peptides. Nevertheless, all promising reaction conditions observed under Method D were further analyzed by Method E employing the better-resolving Aeris peptide column to fully assess regioselectivity.

Consistent with the radiosynthesis of **13**, appreciable incorporation yields for [^{11}C]**2** (65 ± 4.2 % ndc) were observed with at least 12 equivalents of NaOH (Fig. 5) under Method D. However, a closer analysis using Method E revealed inseparable, unidentified radioactive side-products that were diminished under 48 equivalents of base (Fig. 4) with little effect on incorporation yields (59 ± 3.2 % ndc). The major radioactive HPLC peak under the latter conditions ($R_{t(gamma)} = 19.4$ min) coincided with the retention time of the desired *S*-methylated product **2** ($R_{t(gamma)} = 18.9$ min; detector delay $\Delta R_t \approx 0.58$ min), suggesting selective methylation of the cysteine residues in the presence of other competing nucleophilic groups. Qualitatively, the amount of NaOH required appears high, which may suggest the presence of additional low molecular weight protic species, such as residual TFA, in the precursor sample.

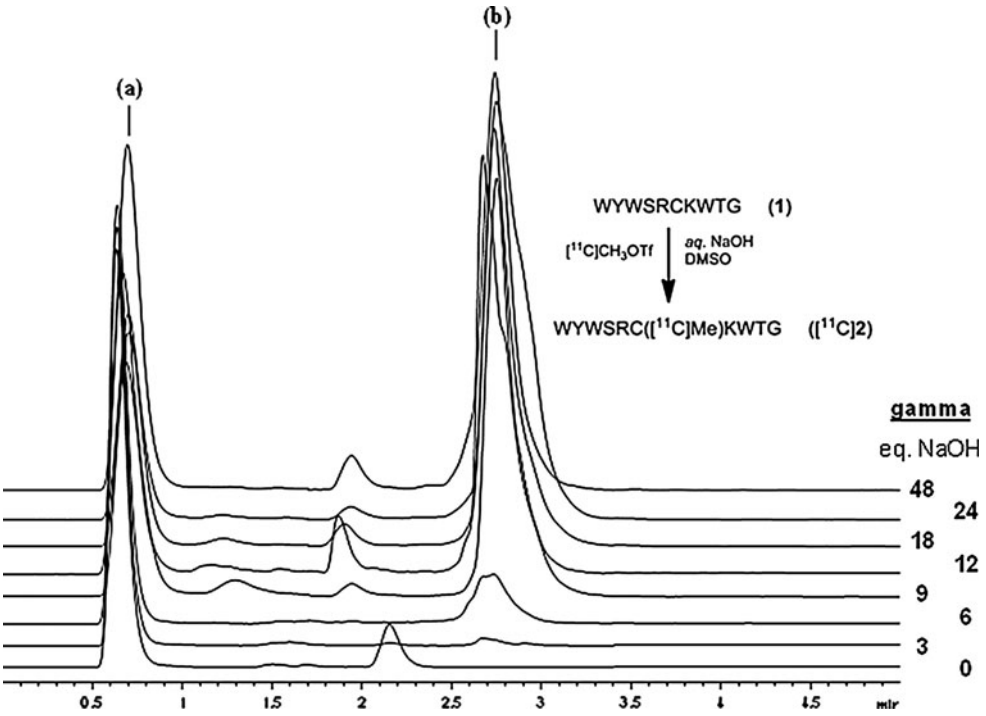

Fig. 5 Stacked radio-HPLC chromatograms (Method D) of the radiosynthesis of [^{11}C]**2** under varying equivalents of NaOH. Reaction conditions: **1** (300 μg), 2.0 M NaOH (0–48 eq), DMSO (300 μL), [^{11}C]MeOTf (185 MBq–1.11 GBq), rt, 20 s. (**a**) [^{11}C]CH_3OH, $R_{t(gamma)} = 0.7$ min. (**b**) [^{11}C]**2**, $R_{t(gamma)} = 2.8$ min

Studies into lowering the quantity of peptide precursor **1** show a 64 ± 3.5 % ndc radiochemical conversion to [^{11}C]**2** as a single radio-HPLC peak starting with 200 μg (0.15 μmol) of peptide **1** (Fig. 6). Lower precursor quantities (<200 μg) show peak broadening suggesting the formation of multiple unidentified radiolabeled products.

Synthesis of [^{11}C]Cys(Me)-[Tyr^3-octreotate] (**9**) and its derivatives **10–12**

Having determined the regioselectivity of [^{11}C]MeOTf rediomethylation towards cysteine residues, this labeling method was extended towards peptide **9**, an octreotate

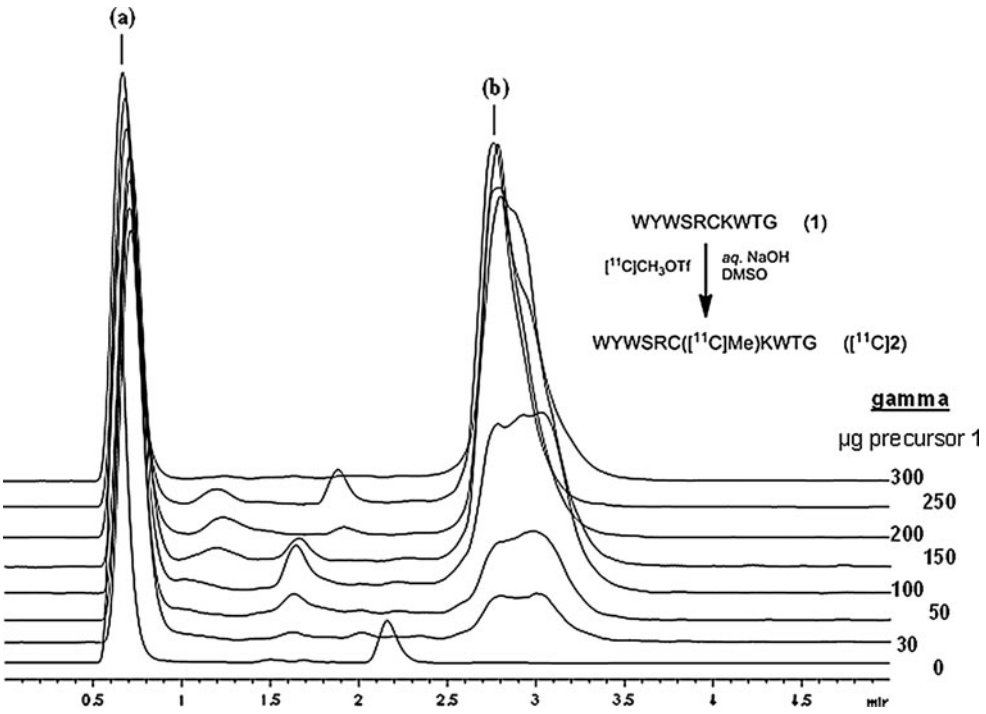

Fig. 6 Stacked radio-HPLC chromatograms (Method D) of the radiosynthesis of [^{11}C]**2** under varying amounts of peptide precursor **1**. Reaction conditions: **1** (0–300 μg), 2.0 M NaOH (48 eq), DMSO (300 μL), [^{11}C]MeOTf (185 MBq–1.11 GBq), rt, 20 s. (**a**) [^{11}C]CH_3OH, $R_{t(gamma)} = 0.7$ min. (**b**) [^{11}C]**2**, $R_{t(gamma)} = 2.8$ min

derivative containing an additional *N*-terminal cysteine residue. The choice for octreotate as model peptide in the current study is two-fold: (1) octreotate and octreotide (an octreotate derivative containing a reduced *C*-terminal threonine residue) are the most successful clinical imaging agents for visualization of somatostatin receptors on *sst*-positive tumors and are currently widely studied as platform for various radioisotopes (Bauer et al. 1982; De Jong et al. 2002; Decristoforo et al. 2003), and (2) chemical modifications at the *N*-terminal end of octreotate do not significantly affect binding affinity of the tracer, but may allow for modulation of its pharmacokinetic properties (Wang et al. 2004). In particular, peptide **9** contains multiple functional groups that are nucleophilic under neutral or basic conditions (Fig. 3). Peptide **9** was synthesized along with the nonradioactive standard **10** via SPPS as described above for peptides **1**–**8**. Of note, the *N*-terminal cysteine building block of **9** was appended following oxidative cyclization by thallium(III) trifluoroacetate to afford the desired oxidation state for the three cysteine residues of the peptide. Due to the cystine functionality of octreotate, derivatives **11** and **12** were synthesized as nonradioactive standards to verify for cystine reduction and subsequent *S*-methylation of these sites under radiolabeling reaction conditions. SPPS yields ranged from 14 to 23 % following semi-preparative HPLC purification under Method B. HPLC Method E proved sufficient to resolve peptides **9**–**12**, and was subsequently employed for analytical and preparative radio-HPLC in the radiosynthesis of [^{11}C]**10** (Fig. 7).

Preparative radiosynthesis of [^{11}C]**10**

Labeled octreotate derivative [^{11}C]**10** was synthesized upon treatment of penta-protic **9** with 40 equivalents of NaOH and [^{11}C]MeOTf. A ca. 52 % ndc radiochemical conversion to the desired product was measured by analytical radio-HPLC without observable traces of [^{11}C]**11** or [^{11}C]**12** from reductive radiomethylation of cystine sites (Fig. 7). Preparative workup involved injection of larger

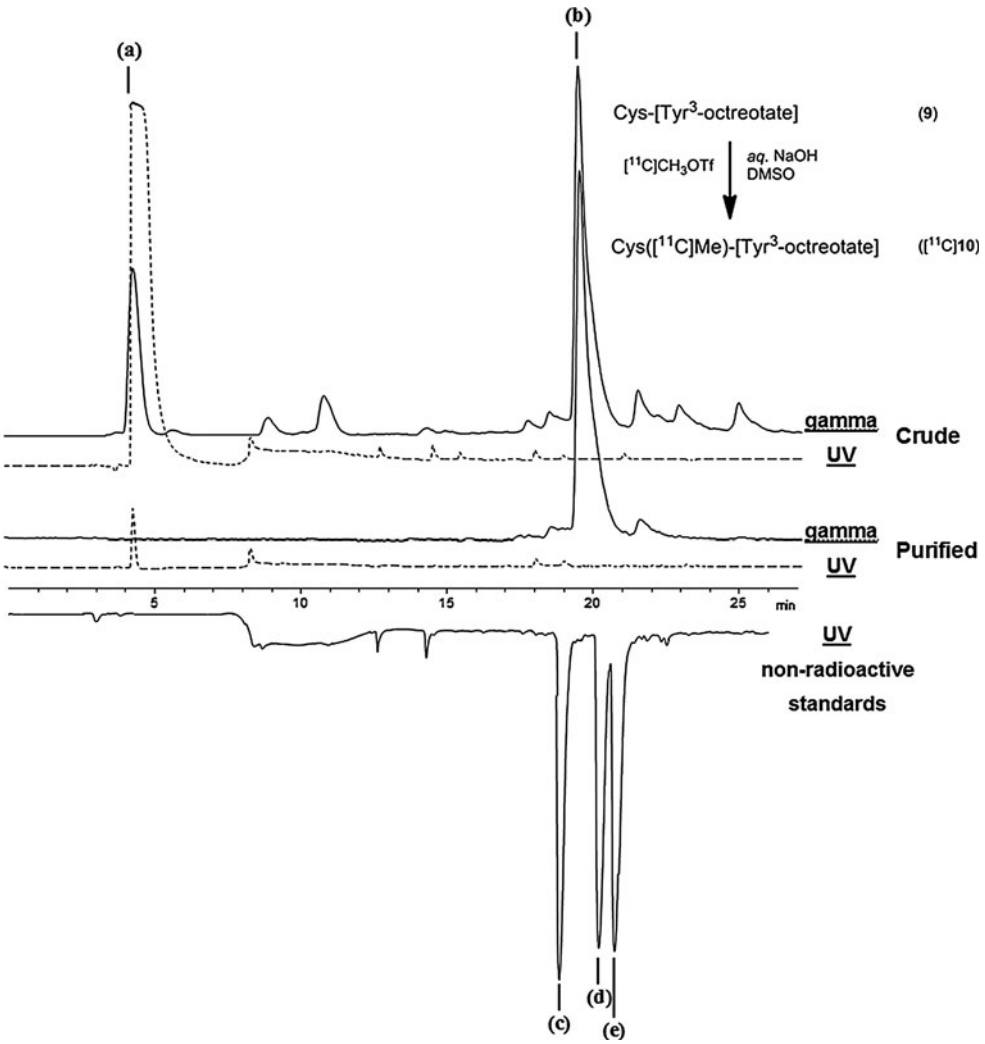

Fig. 7 *Above axis* HPLC radioactive and UV (210 nm) traces of the radiosynthesis of [^{11}C]**10** (Method E). Reaction conditions: **9** (500 μg), 2.0 M NaOH (40 eq), DMSO (300 μL), [^{11}C]MeOTf (370 MBq–4.81 GBq), rt, 20 s. *Below axis* HPLC chromatogram of **10**–**12** (Method E). (**a**) [^{11}C]CH_3OH, $R_{t(gamma)} = 4.3$ min. (**b**) [^{11}C]**10**, $R_{t(gamma)} = 19.4$ min. (**c**) **10**, $R_{t(210\ nm)} = 18.8$ min. (d) **12**, $R_{t(210\ nm)} = 20.2$ min. (e) **11**, $R_{t(210\ nm)} = 20.7$ min. Detector delay $\Delta R_t \approx 0.58$ min at 0.8 mL/min

quantities (ca. 340 MBq) of the reaction medium to the same HPLC setup, collection of the fraction corresponding to the retention time of **10**, and immobilization of the radiolabeled peptide onto C-18 cartridge for removal of MeCN and TFA from HPLC eluent. Formulation of [^{11}C]**10** with ethanol (EtOH) and saline buffer afforded the final product in an overall synthesis time of 30 min and non-decay-corrected RCYs of 11 ± 2 % ($n = 3$). [^{11}C]**10** was isolated with a maximum radioactivity level of 41.8 MBq starting from ca. 340 MBq of [^{11}C]MeOTf. Specific activities (max. 96.3 MBq/μmol) were unimpressive due to inability to separate starting precursor **9** ($R_{t(210\ nm)} =$ 18.0 min) from [^{11}C]**10** ($R_{t(210\ nm)} = 18.8$ min) under the given HPLC method.

Conclusion

We have successfully demonstrated the utility of [^{11}C]MeOTf towards the direct, one-step, and regioselective labeling of peptides at the side-chain of cysteine residues. In particular, the radiosyntheses of [^{11}C]GSMe (**13**) and Trp-Tyr-Trp-Ser-Arg-Cys([^{11}C]Me)-Lys-Trp-Thr-Gly ([^{11}C]**2**) were performed under ambient temperatures (20 °C) and short reaction times, on the order of seconds. In addition, we extend this method towards the synthesis of [^{11}C]Cys(Me)-[Tyr^3-octreotate] as the demonstration of applicability for peptides of biological interest. This octreotate derivative was obtained in non-decay-corrected radiochemical yields of 11 ± 2 % ($n = 3$) with a synthesis time of approx. 30 min. We are currently investigating solutions for addressing the following issues: (1) the stability of peptidic precursors and products under basic labeling conditions, (2) reduction of time of bubbling in the biphasic labeling reaction, e.g. via [^{11}C]MeOTf concentration, (3) reduction of the time of synthesis, especially in the radio-HPLC purification stage, (4) application of labeling method towards other peptides of oncological interest, such as bombesin, cholecystokinin/gastrin, neurotensin, and RGD (Arg-Gly-Asp) (Fani et al. 2012), and finally, (5) the possibility of streamlining the radiochemical process via automation.

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Conflict of interest The authors declare that they have no conflict of interest.

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