Preparation and ^{99m}Tc labeling of hydrazino nicotinamide modified RC-160 conjugate

HU Ji, SUZUKI Kazuko Horiuchi[1], SAJI Hideo[1]

(*Isotope Department, China Institute of Atomic Energy, Beijing 102413;*
[1]*Kyoto University's Graduate School of Pharmaceutical Sciences Department of Patho-Functional Bioanalysis, Kyoto, Japan*)

Abstract Conjugations of 6-Boc-hydrazinopyridine-3-carboxylic acid (Boc-HYNIC) with RC-160 [Lys5, Boc] using different coupling methods were studied. After deprotection, the resulted peptide conjugates were purified by RP-HPLC and characterized by Fab-MS. Combination with different coligands, ^{99m}Tc labeling of HYNIC modified RC-160 conjugate was carried out with high radiolabeling yield (>95%). In vitro stability studies indicated that the final radio-compound remained stable at least up to 20 h when nicotinic acid (NA) used as second coligand (tricine/NA).

Keywords Conjugation, ^{99m}Tc labeling, Somatostatin analogue

CLC numbers O621.3+5, O629.7, R817.9

1 INTRODUCTION

Radiopharmaceuticals based on small peptide have been currently received considerable interests for the development of target specific imaging and therapeutic agents. ^{111}In-DTPA-D-Phe1-octreotide (Octreoscan), a synthetic peptide analogue of the somatostatin (SST), has found a widely clinical application for imaging a range of tumors to date. RC-160 (Vapreotide) is another synthetic analogue of the native SST, which has affinity for a different set of SST sub-receptors and might be used to visualize tumors that do not bind to octreotide, such as exocrine pancreatic tumors and prostate cancer[1].

It has been shown that high labeling yield and specific activity could be achieved using organic hydrazines such as hydrazino nicotinamide (HYNIC) in labeling of protein and small peptides with ^{99m}Tc, and moreover stability and lipophilicity of radiolabeled peptides could be easily adjusted by employing different coligand[2].

Conjugation of Boc-HYNIC to RC-160 [Lys5, Boc] has been carried out in various laboratories using different coupling methods. The yields of the conjugate were, however, reported with wide ranging. Among strategies of peptide syntheses and protein conjugations, the most widely used coupling reagents are carbodiimides (DCC/NHS), and the others are phosphonium and amminium salts. The phosphonium, O-(7-azobenzotriazol-1-yl)-N,N,N',N'-tetra methyl uronium hexafluoro phosphate (HATU), which was introduced by Carpino in 1993[3], has been demonstrated to be more effective in the activation of difficult coupling reaction.

Supported by the IAEA project, CPR/2/008 .
Manuscript received date: 2000-09-20

In this work, conjugations of Boc-HYNIC with RC-160 [Lys5, Boc] were performed with DCC/NHS and HATU in order to compare the suitability and the relative performance as either conjugate method. Radiolabeling of the peptide conjugate with ^{99m}Tc has been carried out in combination with different coligands, and their in vitro stability was also evaluated.

2 EXPERIMENTAL

2.1 Materials

N,N'-dimethyl formamide (DMF), Triethyl amine (TEA), acetonitrile(ACN), trifluoroacetic acid(TFA), N,N'-dicyclohexyl carbodiimide(DCC), diisopropyl ethyl amine(DIEA), thioanisol, nicotinic acid(NA) and tricine were purchased from Nacalai Tesque, Inc., Kyoto. O-(7-azobenzotriazol-1-yl)-N,N,N',N'-tetra methyl uronium hexafluoro phosphate (HATU, 97%) and trisodium triphenylphosphine-3,3',3"-trisulfonate (TPPTS, 96%) were obtained from Aldrich Chemical Co. RC-160[Lys5, Boc] was synthesized by American Peptide Company and supplied through the IAEA, Vienna. All reagents were used as received unless otherwise stated. $Na^{99m}TcO_4$ was eluted in saline solution from a $^{99}Mo/^{99m}Tc$ generator (Daiichi Radioisotope Laboratory, Chiba, Japan). Deionized water was obtained from a Millipore MilliQ Water System and was of >18 MΩ quality.

^{1}H-NMR spectra were recorded on a 270MHz Bruker AC-200 spectrometer, and ^{1}H-NMR data were reported in parts as $\delta(\mu g/g)$ downfield relative to an international standard (TMS). Fast atom bombardment mass (Fab-MS) analysis was performed using a JMS-HX/HX 110 A model (JEOL Ltd., Tokyo), and a mixture of glycerol and NBS as the matrix.

2.2 Instruments and methods

2.2.1 RP-HPLC Reversed-phase high performance liquid chromatography (RP-HPLC) used for analyses and purification were performed on a LC-10AD solvent module (Shimadzu) with SPD-10A UV-VIS detector (Shimadzu) and NaI radiometric detector. HPLC elution profiles were integrated by a D-2500 chromato-integrater (HITACHI). A Cosmosil 5C18-MS column (4.6×150 mm, Nacalai Tesque, Kyoto), flow rate of 1 mL/min and UV detection at 220 nm or 280 nm were employed in combination with the following solvent systems.

Method 1: Solvent (A) 0.1% aqueous TFA/solvent (B) 0.1%TFA in ACN. Gradient: 0-3 min 25% B; 3-23 min 25% to 65% B.

Method 2: Solvent (A) phosphate buffer (0.01mol/L, pH 6.2)/solvent (B) ACN. Gradient: 0-3 min 25% B; 3-23 min 25% to 65% B.

Method 3: Solvent (A) phosphate buffer (0.01mol/L, pH 6.2) / solvent (B) ACN. Gradient: 0-25 min 25% to 35% B; 25-28 min 35% to 50% B; 28-30 min 50% to 75% B.

2.2.2 SPE Purification For purification HYNIC conjugate and Radiolabeled peptides, a solid-phase extraction (SPE) method was used as described in Ref.[4]. Typically,

the solution was passed through a C18 Seppak cartridge (Waters, Milford, MA) previously activated using 5 mL of ethanol, followed by 5 mL of water. The conjugate and radiolabeled complexes were eluted with ACN.

2.3 Syntheses of 6-Boc-HYNIC and 6-Boc-3-NHS-HYNIC

Organic syntheses of 6-Boc-hydrazinopyridine-3-carboxylic acid (6-Boc-HYNIC) and Succinimidyl 6-Boc-hydrazinopyridine-3-carboxylic acid (6-Boc-3-NHS-HYNIC) were carried out according to Abrams' method[5] with minor modification. The final products were characterized by ^{1}H-NMR spectra [6-Boc-HYNIC $\delta(\mu g/g)$: 1.48, 6.69, 8.10, and 8.66; 6-Boc-3-NHS-HYNIC $\delta(\mu g/g)$: 1.42, 2.50, 6.52, 7.96, and 8.58].

2.4 Preparation of HYNIC-RC-160 conjugate

2.4.1 Boc-HYNIC-RC-160 [Lys5, Boc] Conjugation of Boc-HYNIC to RC-160[Lys5, Boc] using different coupling method were carried out as schemed in Fig.1. All the coupling reactions were monitored by HPLC analyses (method 1) and the yields were calculated from the peak areas of Boc-HYNIC- RC-160[Lys5, Boc] and RC-160[Lys5, Boc] analogues.

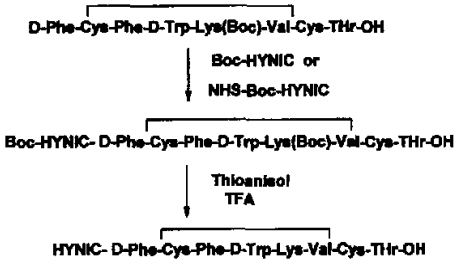

Fig.1 Schematic route of synthesis of HYNIC-RC-160

(1) DCC/NHS method: A total of 2.5 mg RC-160[Lys5, Boc], and 0.8 mg of BOC-3-NHS-HYNIC and 1.2μL of TEA in 60μL DMF were reacted with stirring at room temperature. After 24 h and 46 h the coupling yield by HPLC was 16.43% and 26.74%, respectively. The resulting HYNIC conjugate was initially purified by SPE method and then on HPLC column (method 1). The pure fractions were pooled and lyophilized. Furthermore characterizations were applied on HPLC analysis (method 2) and Fab-MS. Fab-MS m/z $[M+1]^+$ requires: 1467.4, found: 1466.8.

(2) HATU method: A total of 0.50 mg of 6-Boc-HYNIC, 0.62 mg of HATU and 1μL of DIEA in 100μL of DMF were activated for 15 min. A total of 50μL of above reaction solution was added to 1.0 mg of RC-160[Lys5, Boc] in DMF solution and incubated at room temperature. After 1 h the coupling yield by HPLC was almost quantitative and no RC-160[Lys5, Boc] was detected by HPLC. The product was worked up as described above.

2.4.2 HYNIC-RC-160 A total of 1.0 mg of peptide conjugate, 10μL thioanisol, and 200μL TFA were reacted for 15 min at room temperature. After Boc-deprotection the solution was immediately evaporated to dryness under nitrogen flow. The residue was dissolved in 200μL of 50% ethanol and purified on HPLC using method 1. The fractions of HYNIC-RC-160 was pooled and lyophilized. Furthermore characterizations were applied

on HPLC analysis (method 1) and Fab-MS. Fab-MS m/z $[M+1]^+$ requires: 1266.4, found: 1266.6.

2.5 Radiolabeling of HYNIC-RC-160 with ^{99m}Tc

2.5.1 Tricine as coligand To a 2 mL Ependorf vial were added 20μL of HYNIC-RC-160 solution (0.5mg/mL in H_2O), 0.2 mL of tricine solution(100 mg/mL in 0.1mol/L pH 5.0 acetate buffer), 0.2~0.5 mL of $Na^{99m}TcO_4$ solution (10~100 MBq in saline), and 30μL of $SnCl_2{\cdot}2H_2O$(1.0mg/mL in 0.1 mol/L N_2 purged HCl), the reaction mixture was allowed to stand at room temperature for 30 min and analyzed by HPLC.

2.5.2 Tricine and NA as coligands To a 2 mL Ependorf vial were added 20μL of HYNIC-RC-160 solution (0.5mg/mL in H_2O), 0.2 mL of tricine solution(100 mg/mL in 0.1 mol/L pH 5.0 acetate buffer), 0.1 mL of NA (20 mg/mL in 0.1mol/L pH 5.0 acetate buffer), 0.2~0.5 mL of $Na^{99m}TcO_4$ solution (10~100 MBq in saline), and 30μL of $SnCl_2{\cdot}2H_2O$(1.0 mg/mL in 0.1 mol/L N_2 purged HCl). The reaction mixture was heated at 100°C for 30 min and analyzed by HPLC.

2.5.3 Tricine and TPPTS as coligands To a 2 mL Ependorf vial were added 20μL of HYNIC-RC-160 solution (0.5 mg/mL in H_2O), 0.2 mL of tricine solution(100 mg/mL in 0.1mol/L pH 5.0 acetate buffer), 0.1 mL of TPPTS (20 mg/mL in 0.1 mol/L pH 5.0 acetate buffer), 0.2~0.5 mL of $Na^{99m}TcO_4$ solution (10~100MBq in saline), and 30μL of $SnCl_2{\cdot}2H_2O$ (1.0 mg/mL in 0.1 mol/L N_2 purged HCl). The reaction mixture was heated at 100°C for 30~60 min and analyzed by HPLC.

2.6 *In vitro* stability studies

Stability of ^{99m}Tc labeled peptides both in the reaction matrix and in reconstituted solution with phosphate buffer (0.1 mol/L, pH 7.4) after isolated by SPE method from excess ligands were tested by analyses of HPLC at room temperature up to 20 h.

3 RESULT AND DISCUSSION

Conjugations of Boc-HYNIC with RC-160 [Lys5, Boc] were carried out using different coupling reagents, namely DCC/NHS and HATU. The same peptide conjugates were obtained and characterized by Fab-MS. HPLC analyses shown quantitative yield was reached within 1 hr through using HATU, whereas, for DCC/NHS, the yield of 26.74% was reached after 46 h of reaction time. In addition, another alternative protocol, in which the same coupling reagent (HATU) together with Boc-HYNIC and DIEA in the same concentration were added to RC-160 [Lys5, Boc] solution prior to activation, was also undertaken. Although the similar results could be achieved (data not shown), a new side-product for the in situ assembly might be formed. Deprotection of the peptide conjugate was accomplished with TFA and thioanisol (scavenger) in 15 min. The resulted peptide conjugate was purified by RP-HPLC and characterized by Fab-MS. Since the HYNIC group can only occupy one or two sites in the technetium coordination sphere, the use of additional coligands is required. All radiolabeling of HYNIC-RC-160 conju-

gates could be achieved in one step. The radiolabeled peptides were well characterized by two HPLC methods. Using tricine alone as coligand, high radiolabeling yield (>95%) was obtained at room temperature in 30 min. In contrast to tricine, tricine/NA as coligands, radiolabeling need heating in a 100°C water bath for 30min and the yield of >95% could also be achieved. Surprisingly, in case of tricine/TPPTS, very low radiolabeling yield as well as radio multiple peaks were observed when heating the reaction mixture in a 100°C water bath for 30~60 min. Previous studies indicated that the water soluble TPPTS used as second coligand could easily react with HYNIC conjugate in the presence of trcine to produce a ternary complex and then increased stability and minimized number of isomeric forms could be expected[6]. We postulate that it arose as a consequence of steric hindrance existing between HYNIC-RC-160 and bulky TPPTS and /or strong reducing properties of TPPTS through which might disintegrate the disulfide bridge in the peptide.

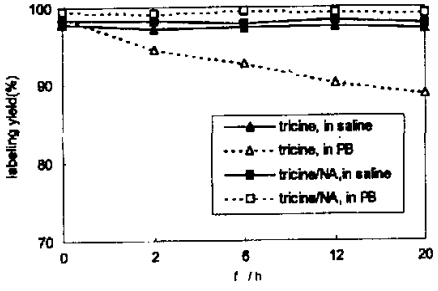

Fig.2 *In vitro* stability of HYNIC-RC-160 labeled with ^{99m}Tc

In vitro stability studies indicated that the final radio-compound remained stable both in the reaction matrix and in reconstituted solution at least up to 20 h for nicotinic acid (NA) used as second coligand (tricine/NA) (see Fig.2). For tricine alone as coligand, lower stability, when removed excess ligands and reconstituted with phosphate buffer (0.1mol/L, pH 7.4), was found. The radiolabeling yield was dropped from 97.8% to 88.7% at room temperature after 20 h.

In conclusion, based on ease of preparation, rate of reaction and coupling yield, HATU is believed to be superior to DCC/NHS in conjugation of Boc-HYNIC with RC-160 [Lys5, Boc]. ^{99m}Tc labeling of HYNIC conjugate could be undertaken in high yields by employing mixture coligands.

Acknowledgement

The authors wish to thank Dr. Ono M. for useful discussions.

References

1 Pinski J, Milovanovic T Y, Yano T *et al.* Proc Soc Exp Bio Med, 1992, **200**:49
2 Ono M, Arano Y, Uehara T *et al.* Bioconjugate Chem, 1999, **10**:386
3 Carpino L A. J Am Chem Soc, 1993, **115**:4397
4 Decristoforo C, and Mather S J. Nucl Med Biol, 1999, **26**:389
5 Abrams M J, Juweid M, tenKate C J *et al.* J Nucl Med, 1990, **31**:2022
6 Edwards E D, Liu S, Zieler M C *et al.* Bioconjugate Chem, 1999, **10**:884