Int J Pept Res Ther (2010) 16:257–266
DOI 10.1007/s10989-010-9227-7

Synthesis of 4-Thiouracil KPGEPGPK Analogues as Potential TIIICBP Identification Tools

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Accepted: 27 July 2010 / Published online: 7 August 2010

Abstract In this paper, we described the synthesis of three octapeptides analogues which are obtained by the incorporation of photochemical 4-thiouracil (s^4U) probes selectively activables. This probe has been incorporated at the 1 and/or 3 positions in KPGEPGPK sequence since it has been demonstrated that those positions could be mutated without major anti-thrombotic activity modifications. These analogues could be used to identify a new receptor for type III collagen named Type III Collagen Binding Protein (TIIICBP).

Keywords 4-Thiouracil probe · TIIICBP · Type III collagen · KPGEPGPK · Anti-thrombotic activity

Abbreviations

TIIICBP	Type III Collagen Binding Protein
s^4U	4-Thiouracil probe
PNA	Peptides nucleic acids
ARN	Ribonucleic acid
NMP	*N*-Methyl-2-pyrrolidone
DIEA	*N*,*N*-Diisopropylethylamine
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3, 3-tetramethyluronium hexafluorophosphate
TFA	Trifluoroacetic acid
Dde	*N*-[1-(4,4-Dimethyl-2, 6-dioxocyclohexylidene)ethyl]
NMM	4-Methylmorpholine
Boc	*tert*-Butyloxycarbonyl
Fmoc	Fluorenylmethyloxycarbonyl
*i*BuOCOCl	Isobutyl chloroformate
FmocOSu	9-Flourenylmethoxycarbonyl-*N*-hydroxysuccinimide
DMSO	Dimethyl sulfoxide

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Introduction

In a previous work, the identification of a new receptor for type III collagen named Type III Collagen Binding Protein (TIIICBP) has been reported (Monnet and Fauvel-Lafève 2000). This receptor which is not yet identified is supposed to interact with the octapeptide sequence KPGEPGPK located in the α1-chain of CB4 fragment of the type III collagen. This octapeptide specifically inhibits platelet interaction with type III collagen in static and flow conditions. This octapeptide prevents in vivo, the thrombosis photochemically-induced in arterioles of mice without affecting bleeding time (Maurice et al. 2006a, b). Actually, the TIIICBP structure and the mechanisms of its corresponding interactions with KPGEPGPK are so far unknown. Our ultimate goal will be to identified TIIICBP (Pires et al. 2007; Pêcher et al. 2009). For this, a solution could be provided by using the 4-thiouracil (s^4U) probe which could be able to form covalent photoadducts, under irradiation, with TIIICBP.

The thionucleobases were used initially as intrinsic probes photoactivated for structural analyzes of the ribonucleic acid (ARN) and to identify the probable contacts between nucleic acids and proteins such as in the complexes nucleoproteins of various systems (replisome, spliceosome, transcription and ribosomes). The thioresidues used (4-thiouracil (s^4U), 4-thiothymine, 6-mercaptopurine, 6-thioguanine) absorb the light in a wavelength of 360 nm. Many works have been done concerning the nature of the

photoadducts formed after s^4U irradiation (Saintomé et al. 1994, 2000; Clivio et al. 1991, 1992a, b). The 4-thiouracil derivatives are also known for their photochemical interactions with different compounds (Fourrey et al. 1973, 1974; Jouin and Fourrey 1975; Fourrey and Moron 1976). However, the greatest interest of this molecule is related to their uses in the synthesis of the oligonucleotides analogues (Saintomé et al. 1994, 2000; Clivio et al. 1991, 1992a, b) and peptides nucleic acids (PNA) (Clivio et al. 1997, 1998; Kosynkina et al. 1994). The peptides nucleic acids were highlighted for the first time by Nielsen and co-workers (Nielsen et al. 1991; Egholm et al. 1992). Since 1991, s everal methods of syntheses, in liquid and solid phases, have been proposed for PNAs (Gasser and Spiccia 2008; Pritz et al. 2006; Upert et al. 2005; Falkiewicz et al. 2001; For reviews see Hyrup and Nielsen 1996; Koppelhus and Nielsen 2003).

In this paper, we described the synthesis of three octapeptides analogues that could be able to bind by a covalent way to TIIICBP via the use of 4-thiouracil (s^4U) probe selectively activable. Those octapeptides analogues are obtained by the incorporation of s^4U, at the 1 and/or 3 positions in KPGEPGPK sequence, since Erickson and colleagues have been demonstrated that those corresponding positions could be mutated without major anti-thrombotic activity modifications (Erickson et al. 1992).

Materials and Methods

The chemicals compounds were purchased from Sigma-Aldrich and used without further purification. Column chromatography was performed on Kielselgel 60 (40–63 μm) ASTM (Merck). Reactions were analyzed on precoated silica gel 60 F_{254} plates (Merck) and the compounds were visualized with a UV lamp (254 nm) and the phosphomolybdic acid reagent and heating. The compounds were analyzed on an analytical reverse-phase HPLC Shimadzu instrument with a prosphere 100 C18 5 μ column (4.6 × 150 mm) using a linear gradient of: (A) 0.1% (v/v) TFA in water and (B) 0.1% (v/v) TFA acetonitrile/water mixture (80/20, v/v), at a flow rate of 1 ml/min with UV detection at 220 nm. Compounds were purified by reverse-phase HPLC using a Shimadzu semi-preparative HPLC system on an prosphere 100 C18 5 μ column (10 × 250 mm) by elution with a linear gradient of (A): aqueous 0.1% TFA and (B): 0.1% (v/v) TFA in acetonitrile/water mixture (80/20), at a flow rate of 3 ml/min with UV detection at 220 nm. The mass spectra and the high-resolution electrospray mass spectra in the positive ion mode were obtained on a Q-TOF Ultima Global hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, U.K.), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound. The source and desolvation temperatures were kept at 80 and 150°C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 50 l/h, respectively. The capillary voltage was 3 kV, the cone voltage 100 V and the RF lens1 energy was optimised for each sample (30–150 V). Lock mass correction, using appropriate cluster ions of an orthophosphoric acid solution (0.1% in 50/50 acetonitrile/water) or of a sodium iodide solution (2 μg/μl in 50/50 propan-2-ol/water + 0.05 μg/μl caesium iodide), was applied for accurate mass measurements. The mass range was typically 50–2,050 Da and spectra were recorded at 2 s/scan in the profile mode at a resolution of 10,000 (FWMH). Data acquisition and processing were performed with MassLynx 4.0 software. Melting points were determined on a Kofler plate and are given uncorrected. Infrared spectra (IR) were recorded on a NICOLET-210 spectrometer using KBr pellets or a Jasco FT/IR 4200. Nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were recorded on a BRUKER AVANCE 500 spectrometer (500 MHz) and tetramethylsilane (TMS) was used as an internal standard. ^1H NMR analyses were obtained at 500 MHz (s: singlet, d: doublet, t: triplet, dd: double doublet, m: multiplet), whereas ^{13}C NMR analyses were obtained at 125 MHz. The chemical shifts (δ) are given in parts per million relative to TMS ($\delta = 0.00$).

Procedure for Preparation of **1a**, **1b** and **1c**

Fmoc(9-fluorophenylmethoxy)-amino acids and Fmoc-amino acids-Sasrin® were purchased from Bachem (Germany). The other chemicals compounds were purchased from Sigma-Aldrich and used without further purification. The peptides were synthesized on an Applied Biosystems Model 433A peptide synthesizer, using standard automated continuous-flow solid-phase peptide synthesis methods. Both manual and automated systems (Applied Biosystem 433A Peptide Synthesizer) were used to prepare the peptides **1a**, **1b** and **1c**. Ten-fold molar excess of the above amino acids were used in a typical coupling reaction. Fmoc-deprotection was accomplished by treatment with a solution of 20% (v/v) piperidine in *N*-methyl-2-pyrrolidone (NMP). The coupling reaction was achieved by treatment with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIEA) in NMP using a standard Fast-Moc protocol. The primary amine protecting group *N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] (Dde) was removed with a solution of 2% hydrazine (v/v) in DMF at room temperature. The peptides were cleaved and side-chain deprotected by treatment of the peptide resin with CH_2Cl_2/TFA (1:9 v/v) during 3 h at room temperature. The octapeptides **1** were purified on an RP-HPLC C18 column (Prosphere® C18, 100 Å 15 μm, 25 × 100 mm) using a

mixture of aqueous 0.1% (v/v) TFA (A) and 0.1% (v/v) TFA in acetonitrile/water mixture (80/20, v/v) (B) as the mobile phase (flow rate of 3 ml/min) and employing UV detection at 220 nm. Electrospray mass spectrometric sequence analysis has been used to confirm the correct sequences (MS–MS, *m/z*).

H_2N-Lys(s^4U)-Pro-Gly-Glu-Pro-Gly-Pro-Lys-OH **1a**

Synthesis of octapeptide started from commercially available Fmoc-Lys(Boc)-Sasrin® **9** (177.0 mg, 0.105 mmol). Then the octapeptide was cleaved from its resin and side chain deprotected by treatment with a mixture of CH_2Cl_2/TFA (1:9 v/v) for 3 h at room temperature. The solid support was removed by filtration, the filtrate concentrated under reduced pressure, and the free octapeptide precipitated from diethyl ether. 27.36 mg of octapeptide **1a** was obtained, as a yellow powder, after reverse-phase chromatography purification (30%). Mp: 187°C. ^{1}H NMR (500 MHz, MeOD), δ (ppm): 1.55 (*m*, 3H, $CH_2\gamma$, Pro), 1.75 (*m*, 3H, $CH_2\gamma$, Pro), 1.79 (*m*, 2H, $CH_2\beta$, Lys), 1.80 (*m*, 4H, $CH_2\delta$, Lys), 1.85 (*m*, 2H, $CH_2\beta$, Glu), 1.90 (*m*, 2H, $CH_2\gamma$, Glu), 2.05 (*m*, 4H, $CH_2\gamma$, Lys), 2.11 (*m*, 2H, $CH_2\beta$, Lys), 3.00 (*m*, 6H, $CH_2\beta$, Pro), 3.30 (*m*, 4H, CH_2^ε, Lys), 3.65 (*m*, 3H, $CH_2\delta$, Pro), 3.76 (*m*, 3H, $CH_2\delta$, Pro), 4.05 (*m*, 4H, CH_2, Gly), 4.29 (*m*, 1H, CHα, Lys), 4.49 (*m*, 1H, CHα, Lys), 4.49 (*m*, 2H, CH_2, s^4U), 4.50 (*m*, 3H, CHα, Pro), 4.80 (*m*, 1H, CHα, Glu), 6.35 (*m*, 1H, CH, s^4U), 7.36 (*m*, 1H, CH, s^4U). ^{13}C NMR (125 MHz, MeOD), δ (ppm): 20.8 ($CH_2\gamma$, Pro), 22.0 ($CH_2\gamma$, Pro, 2C), 24.4 ($CH_2\beta$, Lys), 24.9 ($CH_2\beta$, Lys), 26.1 ($CH_2\beta$, Glu), 26.5 ($CH_2\gamma$, Glu), 29.0 ($CH_2\delta$, Lys), 29.1 ($CH_2\delta$, Lys), 29.2 ($CH_2\beta$, Pro), 29.3 ($CH_2\beta$, Pro, 2C), 29.5 ($CH_2\gamma$, Lys), 30.5 ($CH_2\gamma$, Lys), 38.1 ($CH_2\varepsilon$, Lys), 39.4 (CH_2^ε, Lys), 41.4 (CH_2, Gly), 42.2 (CH_2, Gly), 46.6 ($CH_2\delta$, Pro, 3C), 50.4 (CHα, Glu), 50.5 (CH_2, s^4U), 51.3 (CHα, Lys), 51.7 (CHα, Lys), 60.2 (CHα, Pro), 60.3 (CHα, Pro), 60.9 (CHα, Pro), 112.6 (CH, s^4U), 141.1 (CH, s^4U), 149.2 (CO, s^4U), 167.6 (CO, Gly), 167.7 (CO, Lys), 168.1 (CO, s^4U), 169.9 (CO, Gly), 170.8 (CO, Glu), 172.9 (CO, Pro), 173.0 (CO, Pro), 173.1 (CO, Pro), 173.5 (COOH, Glu), 175.4 (COOH, Lys), 191.4 (CS, s^4U). HRMS $[MH]^+$ calculated for $C_{42}H_{65}N_{12}O_{13}S$: 977.4515, found: 977.4525.

H_2N-Lys-Pro-Lys(s^4U)-Glu-Pro-Gly-Pro-Lys-OH **1b**

Synthesis of octapeptide started from commercially available Fmoc-Lys(Boc)-Sasrin® **9** (169.5 mg, 0.101 mmol). The octapeptide Boc-Lys(Boc)-Pro-Lys(Dde)-Glu(OtBu)-Pro-Gly-Pro-Lys(Boc)-Sasrin® was obtained and the Dde protecting group was manually removed with 2% hydrazine (v/v) in DMF at room temperature. Then the probe 4-thiouracil-1-ylacetic acid **3** was coupled at the N-terminal of the side chain of lysine in position 3 automatically, using a standard Fast-Moc protocol. The completed octapeptide was cleaved from the resin and side chain deprotected by treatment with a mixture of CH_2Cl_2/TFA (1:9 v/v) for 3 h at room temperature. The solid support was removed by filtration, the filtrate concentrated under reduced pressure, and the octapeptide precipitated from diethyl ether to give 15.22 mg of the octapeptide **1b**, as a yellow powder, after reverse-phase chromatography purification (46%). m.p.: 187°C. ^{1}H NMR (500 MHz, CD_3OD), δ (ppm): 1.40 (*m*, 3H, $CH_2\gamma$, Pro), 1.60 (*m*, 3H, $CH_2\gamma$, Pro), 1.65 (*m*, 2H, $CH_2\gamma$, Lys), 1.80 (*m*, 2H, $CH_2\beta$, Glu), 1.80 (*m*, 2H, $CH_2\gamma$, Lys), 1.80 (*m*, 3H, $CH_2\delta$, Lys), 1.90 (*m*, 2H, $CH_2\gamma$, Glu), 2.01 (*m*, 6H, $CH_2\beta$, Lys), 2.07 (*m*, 3H, $CH_2\delta$, Lys), 2.05 (*m*, 2H, $CH_2\gamma$, Lys), 2.49 (*m*, 6H, $CH_2\beta$, Pro), 2.99 (*m*, 4H, CH_2^ε, Lys), 3.10 (*m*, 2H, CH_2^ε, Lys), 3.62 (*m*, 3H, $CH_2\delta$, Pro), 3.80 (*m*, 3H, $CH_2\delta$, Pro), 4.10 (*m*, 2H, CH_2, Gly), 4.22 (*m*, 3H, CHα, Lys), 4.49 (*m*, 2H, CH_2, s^4U), 4.50 (*m*, 3H, CHα, Pro), 4.75 (*m*, 1H, CHα, Glu), 6.36 (*d*, $J = 7.31$ Hz, 1H, CH, s^4U), 7.30 (*d*, $J = 7.37$ Hz, 1H, CH, s^4U). ^{13}C NMR (125 MHz, CD_3OD), δ (ppm): 20.7 ($CH_2\gamma$, Pro), 22.0 ($CH_2\gamma$, Pro), 22.3 ($CH_2\gamma$, Pro), 24.7 ($CH_2\beta$, Glu), 26.0 ($CH_2\beta$, Lys), 26.5 ($CH_2\beta$, Lys), 26.7 ($CH_2\beta$, Lys), 29.1 ($CH_2\delta$, Lys, 3C), 28.3 ($CH_2\gamma$, Glu), 29.2 ($CH_2\beta$, Pro), 29.4 ($CH_2\beta$, Pro), 29.5 ($CH_2\beta$, Pro), 30.5 ($CH_2\gamma$, Lys), 30.6 ($CH_2\gamma$, Lys), 30.8 ($CH_2\gamma$, Lys), 38.7 (CH_2^ε, Lys), 38.9 (CH_2^ε, Lys), 39.4 (CH_2^ε, Lys), 41.4 (CH_2, Gly), 46.5 ($CH_2\delta$, Pro, 3C), 50.4 (CHα, Glu), 51.2 (CH_2, s^4U), 51.4 (CHα, Lys), 53.2 (CHα, Lys, 2C), 60.2 (CHα, Pro, 3C), 112.6 (CH, s^4U), 141.2 (CH, s^4U), 149.2 (CO, s^4U), 167.2 (CO, Gly), 167.6 (CO, Lys), 168.2 (CH_2-CO, s^4U), 170.9 (CO, Glu), 171.1 (CO, Lys), 172.7 (CO, Pro), 172.9 (CO, Pro), 173.1 (CO, Pro), 173.5 (COOH, Glu), 175.1 (COOH, Lys), 191.3 (CS, s^4U). HRMS $[MH]^+$ calculated for $C_{46}H_{74}N_{13}O_{13}S$: 1048.5250, found: 1048.5275.

H_2N-Lys(s^4U)-Pro-Lys(s^4U)-Glu-Pro-Gly-Pro-Lys-OH **1c**

Synthesis of octapeptide started from commercially available Fmoc-Lys(Boc)-Sasrin® **9** (182.24 mg, 0.108 mmol). The octapeptide Boc-Lys(Fmoc)-Pro-Lys(Dde)-Glu(OtBu)-Pro-Gly-Pro-Lys(Boc)-Sasrin® **10** was obtained and Fmoc-deprotection was accomplished by treatment with a solution of 20% (v/v) piperidine in NMP. The probe 4-thiouracil-1-ylacetic acid **3** was coupled at the N-terminal of the side chain of lysine in position 1, using a standard Fast-Moc protocol. The octapeptide Boc-Lys(s^4U)-Pro-Lys(Dde)-Glu(OtBu)-Pro-Gly-Pro-Lys(Boc)-Sasrin® was obtained and Dde protecting group was manually removed with a solution of 2% hydrazine (v/v) in DMF at room temperature. A second probe 4-thiouracil-1-ylacetic acid **3** was coupled at the N-terminal of the side chain of lysine in position 3 automatically, using a standard Fast-Moc protocol. The completed octapeptide was cleaved from the resin and side

chain deprotected by treatment with a mixture of CH_2Cl_2/TFA (1:9 v/v) for 3 h at room temperature. The solid support was removed by filtration, the filtrate concentrated under reduced pressure. Peptide was purified using reverse-phase chromatography to afford 20.22 mg of octapeptide **1c** as a yellow powder (26%). m.p.: 188°C. HRMS $[MH]^+$ calculated for $C_{52}H_{77}N_{15}O_{15}S_2$: 1215.5165, found: 1215.5109.

Fmoc-Lys(s^4U)-OH **2**

Sodium carbonate (5.5 mg, 65.47 μmol) and FmocOSu (6.4 mg, 18.97 μmol) in dioxane (5 ml) were added to a solution of H_2N-Lys(s^4U)-OH **8** (5 mg, 15.87 μmol) in water (5 ml). The reaction mixture was stirred overnight at room temperature. The reaction was cooled to 0°C and acidified to pH 3 with HCl 9 N. The product was extracted with EtOAc (3 × 30 ml). The organic phase was washed with water, dried ($MgSO_4$) and evaporated. After preparative HPLC purification, 5.13 mg of Fmoc-Lys(s^4U)-OH **2** was obtained as a yellow oil (60%). ^{1}H NMR (500 MHz, CD_3OD), δ (ppm): 1.40 (*m*, 2H, $CH_2\beta$, Lys), 1.50 (*m*, 2H, $CH_2\delta$, Lys), 1.90 (*m*, 2H, $CH_2\gamma$, Lys), 3.21 (*m*, 2H, $CH_2\varepsilon$, Lys), 3.60 (*m*, 1H, CHα, Lys), 3.80 (*m*, 2H, OCH_2, Fmoc), 3.80 (*m*, 1H, CH, Fmoc), 4.40 (*s*, 2H, CH_2, s^4U), 6.35 (*d*, $J = 7.44$ Hz, 1H, CH, s^4U), 7.20 (*m*, 2H, CH, Fmoc), 7.25 (*m*, 2H, CH, Fmoc), 7.25 (*m*, 1H, CH, s^4U), 7.60 (*m*, 2H, CH, Fmoc), 7.80 (*m*, 2H, CH, Fmoc). ^{13}C NMR (125 MHz, CD_3OD), δ (ppm): 22.8 ($CH_2\beta$, Lys), 25.0 ($CH_2\delta$, Lys), 28.4 ($CH_2\gamma$, Lys), 36.6 (CH_2^{ε}, Lys), 49.0 (CH_2, s^4U), 50.2 (CH, Fmoc), 52.0 (CHα, Lys), 66.6 (OCH_2, Fmoc), 112.9 (CH, s^4U), 119.6 (CH, Fmoc, 2C), 124.8 (CH, Fmoc, 2C), 126.7 (CH, Fmoc, 2C), 127.4 (CH, Fmoc, 2C), 140.3 (CH, s^4U), 141.2 (Cq, Fmoc, 2C), 143.9 (Cq, Fmoc, 2C), 148.9 (CO, s^4U), 168.1 (CO, s^4U), 169.2 (CO, Fmoc), 173.4 (COOH, Lys), 191.3 (CS, s^4U). MS $[MNa]^+$ $C_{27}H_{28}N_4O_6SNa$ found: 559.23 (100%, molecular peak).

2-(2-Oxo-4-thioxo-5,6-dihydro-3*H*-pyrimidin-1-yl)-ethanoic acid **3**

A suspension of **5** (8 g, 40.0 mmol) in 2 N NaOH aqueous (50 ml) was sonicated for 30 min. The reaction was cooled to 0°C and acidified to pH 2 with HCl 9 N. The formed precipitate was filtrated, washed with cold water, and dried at room temperature in a dessicator over $CaCl_2$ under vacuum yielding 4.84 g of **3** as a slightly yellow powder (65%). m.p.: 220°C. ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 4.40 (s, 2H, CH_2), 6.29 (d, $J = 6.85$ Hz, 1H, CH), 7.52 (d, $J = 6.85$ Hz, 1H, N–CH), 12.65 (s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm): 49.7 (CH_2), 112.5 (N–CH), 142.1 (CH), 148.9 (CO), 169.4 (COOH), 191.2 (CS). HRMS $[MNa]^+$ calculated for $C_6H_6N_2O_3S^{23}Na$: 208.9997, found: 209.0006.

2-(2,4-Dioxo-5,6-dihydro-3H-pyrimidin-1-yl)-methyl ethanoate **4**

A suspension of uracil (10 g, 89.2 mmol) and K_2CO_3 (30.82, 223.03 mmol) in water (100 ml) was stirred at 80°C for 15 min. A solution of methyl 2-bromoethanoate (20.4 g, 133.3 mmol) in water (10 ml) was added and the solution was stirred at 80°C for 1 h. After cooling, the precipitate was filtered, washed with cold water and dried at room temperature in a desiccator, in the presence of $CaCl_2$ and under vacuum, to give 10 g of **4** as a white powder (61%). m.p.: 180°C. IR (KBr, cm^{-1}): 3136 (NH), 1700 (CO). ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 3.68 (s, 3H, OCH_3), 4.55 (s, 2H, CH_2), 5.56 (d, $J = 7.60$ Hz, 1H, CH), 7.55 (d, $J = 7.60$ Hz, 1H, N–CH), 11.40 (s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm): 49.0 (CH_2), 52.7 (OCH_3), 101.6 (N–CH), 146.3 (CH), 151.4 (CO–N), 164.1 (CO–CH), 169.1 (COO). MS $[MNa]^+$ $C_7H_8N_2O_4Na$ found: 207.04 (100%, molecular peak).

2-(2-Oxo-4-thioxo-5,6-dihydro-3H-pyrimidin-1-yl)-methyl ethanoate **5**

To a boiling solution of **4** (10 g, 54.3 mmol) in dioxane (100 ml) was added P_2S_5 (14.42 g, 65.2 mmol). The solution was stirred for 2 h and then filtered on Celite®. The filtrate was then concentrated to dryness. After purification by silica gel chromatography give, 15 g of **5** were obtained as a yellow powder (69%). m.p.: 180°C. ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 3.68 (*s*, 3H, OCH_3), 4.55 (*s*, 2H, CH_2), 6.32 (d, $J = 7.33$ Hz, 1H, CH), 7.53 (d, $J = 7.33$ Hz, 1H, N–CH), 12.80 (s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm): 49.7 (CH_2), 52.9 (OCH_3), 112.8 (N–CH), 141.9 (CH), 148.8 (CO), 168.5 (COO), 191.3 (CS). MS $[MH]^+$ $C_7H_9N_2O_3S$ found: 201.03 (100%, molecular peak).

Boc-Lys(s^4U)-OCH_3 **6**

4-Thiouracil-1-ylacetic acid **3** (2.95 g, 15.89 mmol), NMM (1.74 g, 15.89 mmol), and *i*BuOCOCl (2.17 g, 15.89 mmol) were dissolved in CH_2Cl_2 (25 ml). After few minutes, triethylamine (1.6 g, 15.89 mmol) was added, followed by an addition of a solution of Boc-Lys-OCH_3 (3.72 g, 14.30 mmol) in CH_2Cl_2 (25 ml). The reaction was stirred for 30 min at room temperature. The solvent was then evaporated at reduced pressure, and the residue was purified on silica gel with ethyl acetate (100%) to give 5.22 g of **6** as a yellow powder (81%). m.p.: 128°C. ^{1}H NMR (500 MHz, CD_3OD), δ (ppm): 1.35 (s, 9H, CH_3, Boc), 1.30 (m, 2H,

$CH_2\beta$, Lys), 1.47 (m, 2H, $CH_2\delta$, Lys), 1.72 (m, 2H, $CH_2\gamma$, Lys), 3.19 (m, 2H, CH_2^{ε}, Lys), 3.65 (s, 3H, OCH_3), 4.03 (m, 1H, CHα, Lys), 4.41 (s, 2H, CH_2, s^4U), 6.31 (d, $J = 7.32$ Hz, 1H, CH, s^4U), 7.39 (d, $J = 7.32$ Hz, 1H, CH, s^4U), 8,35 (s, 1H, NH, Lys). ^{13}C NMR (125 MHz, CD_3OD), δ (ppm): 22.7 ($CH_2\beta$, Lys), 27.3 (CH_3, Boc), 28.4 ($CH_2\delta$, Lys), 30.8 ($CH_2\gamma$, Lys), 39.0 (CH_2^{ε}, Lys), 50.3 (CH_2, s^4U), 51.2 (OCH_3), 53.6 (CHα, Lys), 79.2 (Cq, Boc), 112.5 (CH, s^4U), 140.9 (CH, s^4U), 149.1 (CO, s^4U), 156.7 (CO, Boc), 167.3 (CO, s^4U), 173.6 (COO, Lys), 191.4 (CS, s^4U). HRMS $[MNa]^+$ calculated for $C_{18}H_{28}N_4O_6S^{23}Na$: 451.1627, found: 451.1631.

Boc-Lys(s^4U)-OH **7**

A solution of Boc-Lys(s^4U)-OCH_3 (1 g, 2.33 mmol) in methanol (25 ml) was added an aqueous solution of 10% NaOH (25 ml). The reaction was stirred for 2 h at 0°C. The organic solvent was then evaporated at reduced pressure, and the solution was neutralized using excess HCl. The product was extracted in EtOAc (3 × 30 ml). The organic phase was washed with water, dried ($MgSO_4$) and evaporated to furnish 724 mg of **7** as a yellow powder (75%). m.p.: 130°C. ^1H NMR (500 MHz, CD_3OD), δ (ppm): 1.39 (*s*, 9H, CH_3, Boc), 1.40 (*m*, 2H, $CH_2\beta$, Lys), 1.45 (*m*, 2H, $CH_2\delta$, Lys), 1.75 (*m*, 2H, $CH_2\gamma$, Lys), 3.25 (*m*, 2H, $CH_2\varepsilon$, Lys), 4.09 (*m*, 1H, CHα, Lys), 4.43 (*s*, 2H, CH_2, s^4U), 6.35 (*d*, $J = 7.20$ Hz, 1H, CH, s^4U), 7.38 (*d*, $J = 7.20$ Hz, 1H, CH, s^4U). ^{13}C NMR (125 MHz, CD_3OD), δ (ppm): 22,8 ($CH_2\beta$, Lys), 27.3 (CH_3, Boc), 28.4 ($CH_2\delta$, Lys), 30.0 ($CH_2\gamma$, Lys), 38.9 (CH_2^{ε}, Lys), 50.2 (CH_2, s^4U), 53.4 (CHα, Lys), 79.1 (Cq, Boc), 112.5 (CH, s^4U), 140.9 (CH, s^4U), 149.1 (CO, s^4U), 156.8 (COO, Boc), 167.3 (CO, s^4U), 174.8 (COO, Lys), 191.5 (CS, s^4U). HRMS $[MH]^+$ calculated for $C_{17}H_{26}N_4O_6S$: 437.1471, found: 437.1462.

H_2N-Lys(s^4U)-OH **8**

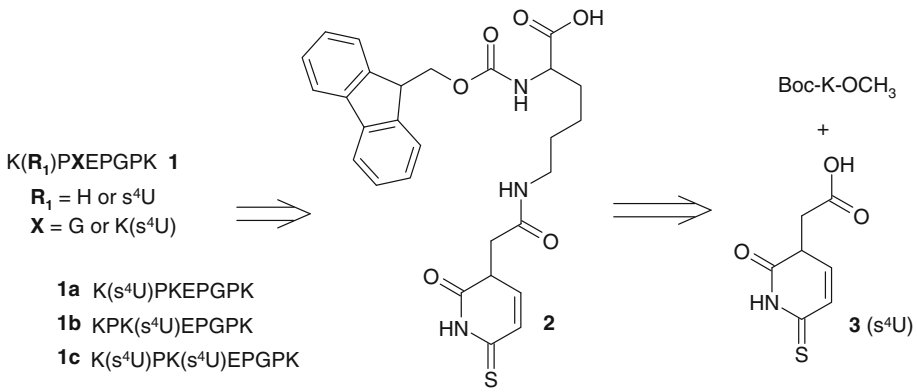
Boc-Lys(s^4U)-OH **7** (20 mg, 45.75 µmol) was cleaved by TFA treatment (10%) in CH_2Cl_2 (5 ml) during 3 h at room temperature. The solvent was then evaporated at reduced pressure, and the residue was washed with ether to give 16.7 mg of **8** as a yellow powder (87%). m.p.: 130°C. ^1H NMR (500 MHz, CD_3OD), δ (ppm): 1.37 (*m*, 2H, $CH_2\beta$, Lys), 1.59 (*m*, 2H, $CH_2\delta$, Lys), 1.85 (*m*, 2H, $CH_2\gamma$, Lys), 3.19 (*m*, 2H, CH_2^{ε}, Lys), 3.65 (*m*, 1H, CHα, Lys), 4.33 (*s*, 2H, CH_2, s^4U), 6.20 (*d*, $J = 6.81$ Hz, 1H, CH, s^4U), 7.18 (*d*, $J = 6.81$ Hz, 1H, CH, s^4U). ^{13}C NMR (125 MHz, CD_3OD), δ (ppm): 20.3 ($CH_2\beta$, Lys), 25.1 ($CH_2\delta$, Lys), 28.4 ($CH_2\gamma$, Lys), 37.4 (CH_2^{ε}, Lys), 49.0 (CH_2, s^4U), 52.1 (CHα, Lys), 111.1 (CH, s^4U), 139.4 (CH, s^4U), 147.7 (CO, s^4U), 166.0 (CO, s^4U), 170.5 (COOH, Lys), 189.9 (CS, s^4U). MS $[MH]^+$ $C_{12}H_{19}N_4O_4S$ found: 315.29 (100%, molecular peak).

Results and Discussion

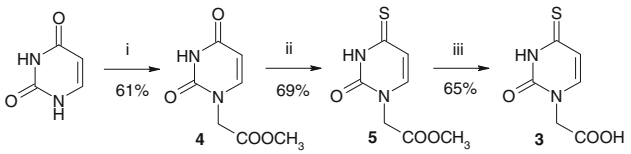
In order to obtain the peptides **1**, the 4-thiouracil probe at the N-terminal of the side chain of the lysine could be incorporated, according to a Fmoc chemistry strategy, in the position 1 and/or 3 into the reference octapeptide KPGEPGPK sequence. The three corresponding octapeptides **1a–c** could be obtained from the modified lysine **2** starting from the 4-thiouracil-1-ylacetic acid **3** (s^4U) (Scheme 1).

The synthesis of the simple derivative 4-thiouracil **3** is based on a methodology initially described by Wheeler and Liddle (1908) and modified by Egholm et al. (1992). In our case, the probe **3** was prepared in three steps from the commercially available uracil (Scheme 2) (Clivio et al. 1998; Egholm et al. 1992).

Treatment of uracil with methyl 2-bromoethanoate and K_2CO_3, as a base, furnished **4** in 61% yield (Scheme 2). ^{13}C NMR of **4** indicates a signal at 49.0 ppm corresponding to the methylenic group and 52.7 ppm to the methyl group. This compound treated with a phosphorus pentasulfide solution under boiling solution affords the sulfur compound **5**. The spectra ^{13}C NMR confirm the appearance of a signal



Scheme 1 Retrosynthesis of octapeptides analogues **1**



Scheme 2 Synthesis of 4-thiouracil-1-ylacetic acid **3**. Reagents: (i) K_2CO_3, $BrCH_2COOCH_3$, DMF; (ii) P_2S_5, dioxane, reflux; (iii) NaOH, water

at 191.3 ppm corresponding to the C=S group. Finally, the compound **5** was saponified to yield the corresponding s^4U **3** (total yield 29%). These reactions containing the 4-thiouracil must, of course, be realized safe from the light. ^{13}C NMR of the acid **3** confirms the presence of the thioamide group represented by the signal at 191.2 ppm and the liaison CH=CH by the signal at 112.5 and 142.1 ppm (Fig. 1). We can also check the presence of the acid group by the signal at 169.4 ppm. The high-resolution electrospray mass spectra of **3** show the molecular ion at 209.0006 $[MNa]^+$.

The probe lysine Fmoc **2** was synthesized by two different methods (Scheme 3). First, the lysine **2** synthesis was carried out by a direct coupling reaction between the Fmoc-Lys-OH and the probe s^4U **3** in the presence of

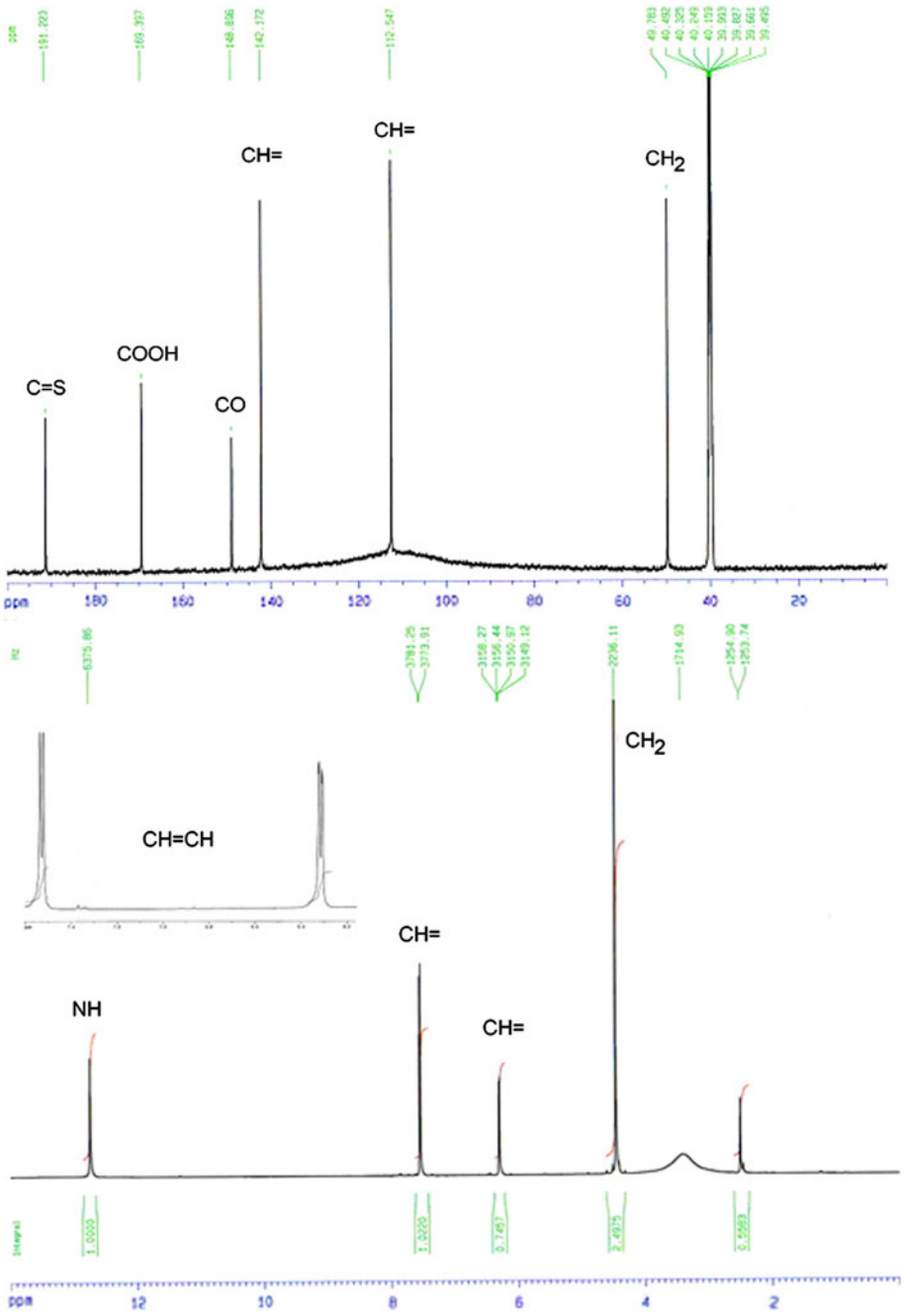


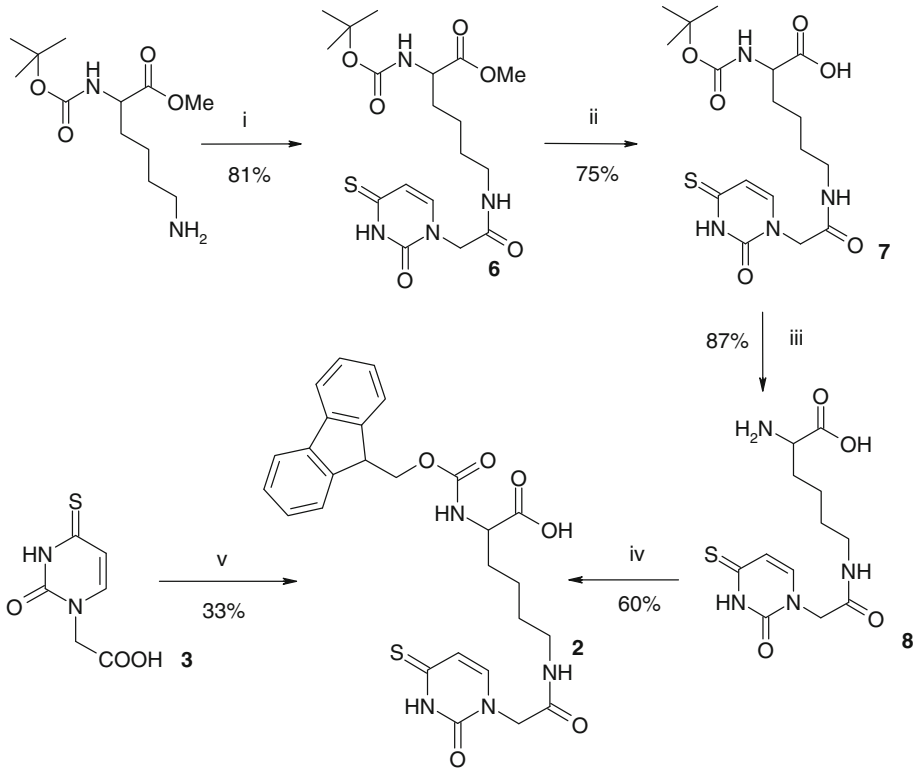
Fig. 1 ^{13}C NMR and ^1H NMR (DMSO-d_6) spectrum of probe **3**

*i*BuOCOCl in CH_2Cl_2. During this reaction, many impurities have been formed and observed by HPLC and the corresponding lysine **2** was obtained in low yield (33% HPLC yield). Moreover, the retention times of these different compounds being too close, HPLC purification was so constraining and unfortunately prevented us from working with significant amounts.

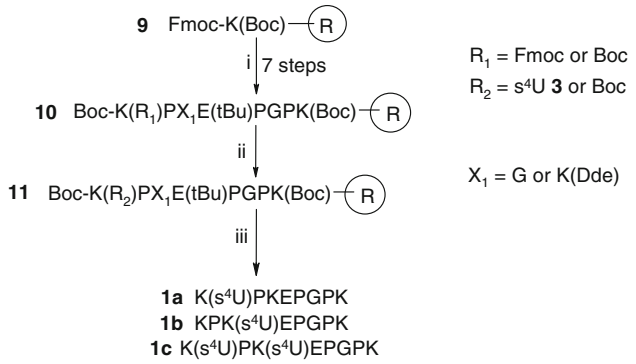
Consequently, a second new way has been used in which the probe was incorporated at the beginning of the synthesis. For this, the probe lysine protected **2** was obtained in four steps starting from the Boc-Lys-OCH_3 (Scheme 3). The coupling reaction with the protected lysine and the probe s^4U **3** was accomplished in the presence of *i*BuOCOCl/NMM to afford **6** (81%). The corresponding ^1H NMR spectra shows the presence of the nine protons of Boc group at 1.35 ppm and two protons ethylene CH=CH of the probe at 6.31 ppm and 7.39 ppm. In NMR ^{13}C, the presence of carbons related to these two CH protons were observed at 112.5 and 140.9 ppm. The compound **6** was then saponified to give **7** as 75% yield. After Boc deprotection of **5**, the corresponding lysine **8** was engaged with FmocOSu to afford compound **2**, after preparative HPLC purification (60% yield). ^1H NMR showed the signal characteristic of Fmoc group at 7.20, 7.25, 7.60 and 7.80 ppm. In this pathway synthesis (31% total yield), the photoactivable probe remains stable during all the synthesis, in particular, in the acidic conditions used to obtain the deprotected lysine **8**. In the two methods used, we always observed impurities generally formed in the coupling reaction. This unfortunately prevents us from working with significant amounts of the probe **2**.

Consequently, to limit the formation of secondary products; we have decided to directly incorporate the probe **3** into the lysine in position 1 and/or 3 at the end of the peptide synthesis. For that, the octapeptide Fmoc synthesis is carried out starting from the amino acid L-Fmoc-Lys(Boc) grafted with the Sasrin® resin in C-terminal. The different amino acids are incorporated by peptide coupling using HBTU/DIEA. At each step, the Fmoc group is cleaved using a 20% piperidine solution in the NMP. The difficulty here consist to incorporate a lysine into 1 and/or 3 position with a selectively protective group (Dde, Fmoc, Boc) grafted in the function amine N^{ε} of the side chain. After synthesis of the octapeptide, the side chain amino function of the lysine in position 1 and/or 3 is selectively cleaved to be coupled with the probe thiouracil **3** (s^4U).

The octapeptide K(s^4U)PGEPGPK **1a** is synthesized starting from Fmoc-K(Boc)-Sasrin® resin **9** (Scheme 4). The seven amino acids are introduced into a sequential order in fourteen steps to obtain the correctly protected octapeptide **10** (R_1 = Fmoc, X_1 = G). The last added amino acid is a Boc-Lys(Fmoc)-OH. The Fmoc group was cleaved selectively using a 20% piperidine solution in NMP. Consequently, only the function amine N^{ε} of the side chain of lysine is free. The probe **3** was then coupled to this octapeptide



Scheme 3 Synthesis of amino acid **2**. Reagents: (i) 4-thiouracil-1-ylacetic acid **3**, iBuOCOCl, NMM, TEA, CH_2Cl_2; (ii) NaOH, MeOH, water; (iii) TFA 10%, CH_2Cl_2; (iv) FmocOSu, $NaHCO_3$, dioxane, water; (v) Fmoc-K-OH, iBuOCOCl, NMM, TEA, CH_2Cl_2



Scheme 4 Synthesis of octapeptide analogues **1a**, **1b** and **1c**. Reagents: (i) Fmoc-amino acids, HBTU; (ii) R_1 = Fmoc; X_1 = G: (a) piperidine 20%, NMP; (b) 4-thiouracil-1-ylacetic acid **7**, HBTU; R_1 = Boc; X_1 = K(Dde): (a) NH_2NH_2 2%, DMF; (b) 4-thiouracil-1-ylacetic acid **3**, HBTU; R_1 = Fmoc; X_1 = K(Dde): (a) piperidine 20%, NMP; (b) 4-thiouracil-1-ylacetic acid **3**, HBTU; (c) NH_2NH_2 2%, DMF; (d) 4-thiouracil-1-ylacetic acid 3, HBTU; (iii) TFA 90%, CH_2Cl_2

function using HBTU/DIEA to give the corresponding octapeptide **11** (R_2 = s^4U, X_1 = G) grafted on the resin and incorporating the probe. Lastly, after cleavage of the protective groups and the resin, using a 90% TFA solution in CH_2Cl_2, the octapeptide K(s^4U)PGEPGPK **1a** is obtained, after purification by preparative HPLC, with a total yield of 30%.

Peptide K(s^4U)PGEPGPK **1a** was characterized by the mass and NMR spectroscopic analysis. The molecular ion $[MH]^+$ is represented by the peak at 977.4 on the mass spectra (Fig. 2). The mass spectra shows peptide fragments at 681.4 (ion y7, PGEPGPK), 584.3 (ion y6, GEPGPK), 527.3 (ion y5, EPGPK), 398.2 (ion y4, PGPK), 301.2 (ion y3, GPK), 244.2 (ion y2, PK) and 147.1 (ion y1, K). Only some ions b, corresponding to the loss of amino acids on the N-terminal side have been observed at 580.2 (ion b4, K(s^4U)PGE), 451.2 (ion b3, K(s^4U)PG) and 394.1 (ion b2, K(s^4U)P). Some other fragments (ions b) are also observables corresponding with peptide fragments in which the probe is missing on the level of the side chain of lysine: 226.1 (ion KP) and 509.2 (ion KPGEP).

^{13}C NMR spectra shows the corresponding carbonyl peaks between 149 and 175 ppm: 149.2 (s^4U), 167.6 (G), 167.7 (K), 168.1 (s^4U), 169.9 (G), 170.8 (E), 172.9 (P), 173.0 (P), 173.1 (P), 173.5 (E) and 175.4 ppm (K). The signal at 191.4 ppm confirms the presence of the probe group C=S. The two probe ethylene carbons are present at 141.1 and 112.6 ppm. ^{1}H NMR spectra shows the probe ethylene protons at 6.42 ppm and 7.38 ppm. The α amino acids protons are present at 4.05 (G), 4.29 (K), 4.49 (K), 4.50 (P) and 4.80 ppm (E).

The second octapeptide modified KPK(s^4U)EPGPK **1b** was synthesized according to the same protocol synthesis such as octapeptide **1a** previously described. The lysine which incorporated the probe possesses a Fmoc α-amino function protection whereas the side chain amino function is protected with a Dde group (Scheme 4). In the case of the lysine in position 1, in which no probe is incorporated, its two amino functions are Boc protected. After introduction of the seven corresponding amino acids, starting from the Fmoc-Lys(Boc)-Sasrin® resin **9**, the correctly protected octapeptide **10** (R_1 = Boc, X_1 = K(Dde)) is obtained. The cleavage of the Dde group (side chain of the lysine located in position 3) is carried out manually (i.e. out of automatic synthetizer) by the addition of a 2% hydrazine

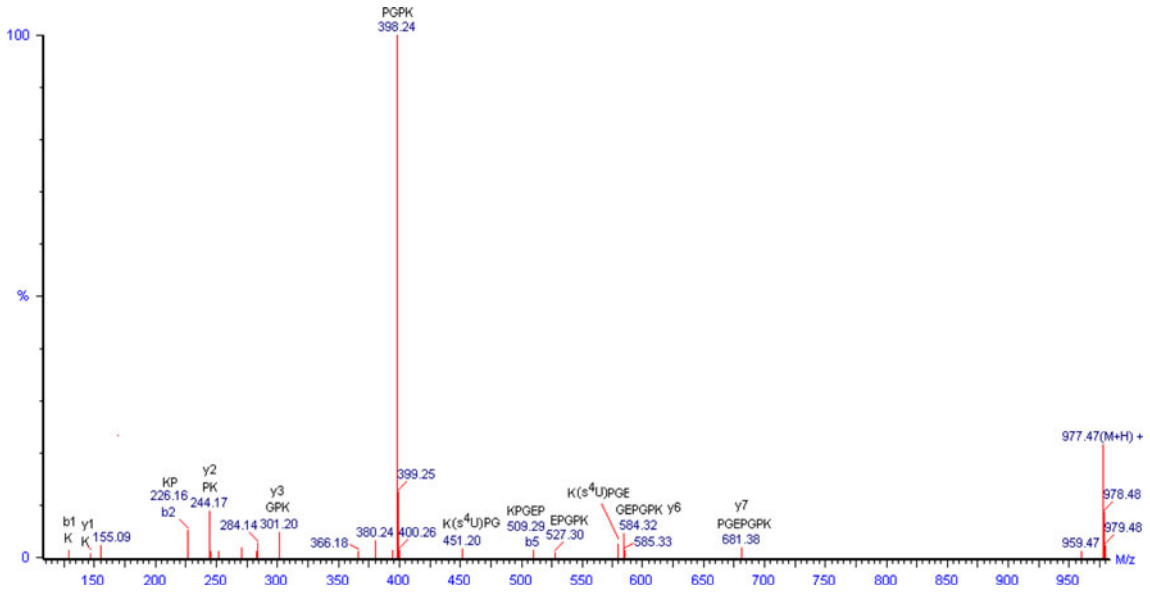


Fig. 2 MS spectrum of peptide K(s^4U)PGEPGPK **1a**

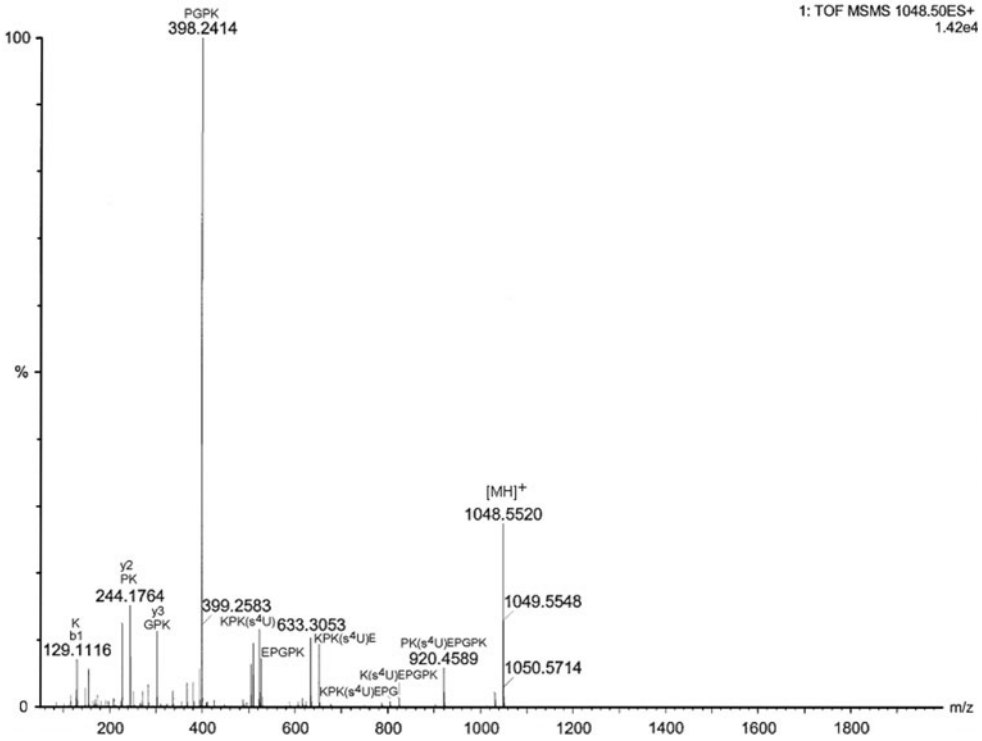


Fig. 3 MS spectrum of peptide KPK(s^{4}U)EPGPK **1b**

solution in DMF. The corresponding peptide is then coupled with the probe **3** using HBTU/DIEA to afford the octapeptide KPK(s^{4}U)EPGPK **1b** with a total yield of 46%, after cleavage of the protective groups (Boc) and the resin using a solution 90% TFA solution in CH_2Cl_2 and preparative HPLC purification. The two octapeptides **1a** and **1b** present similar mass spectra (Fig. 3). The molecular ion **1b** is observed at 1048.5 $[MH]^+$. The fragments corresponding to the ions b are represented by the masses at 506.9 (ion b6, KPK(s^{4}U)EPG), 652.7 (ion b4, KPK(s^{4}U)E), 523.6 (ion b3, KPK(s^{4}U)) and 129.1 (ion b1, K). Other peptide fragments are seen (ions y) at 920.4 (ion y7, PK(s^{4}U)EPGPK), 822.9 (ion y6, K(s^{4}U)EPGPK), 527.3 (ion y5, EPGPK), 398.2 (ion y4, PGPK), 301.2 (ion y3, GPK) and 244.1 (ion y2, PK).

The last octapeptide modified **1c** possesses two probes incorporated in the two lysine side chains located into positions 1 and 3. The lysine in position 3 has a (-amino function Fmoc protected and a side chain amino function Dde protected (Fmoc-Lys(Dde)). The lysine in position 1 possesses a α-amino function Boc and a side chain amino function Fmoc protected (Boc-Lys(Fmoc)) (Scheme 4). From L-Fmoc-Lys(Boc) grafted on the resin **9**, the sequential introduction of seven amino acids was carried out. Then, after the side chain lysine deprotection (Fmoc) by using piperidine, the probe **3** is coupled. The Dde group (lysine in position 3) is cleaved manually using a 2% hydrazine solution in DMF. The second probe **3** was then incorporated by peptide coupling using HBTU. Lastly, after final deprotection and resin cleavage, the octapeptide K(s^{4}U)PK(s^{4}U)EPGPK **1c** is obtained with a total yield of 26% after purification on preparative HPLC.

The octapeptide K(s^{4}U)PK(s^{4}U)EPGPK **1c** was identified by spectroscopy of mass (Fig. 4). The peak corresponding

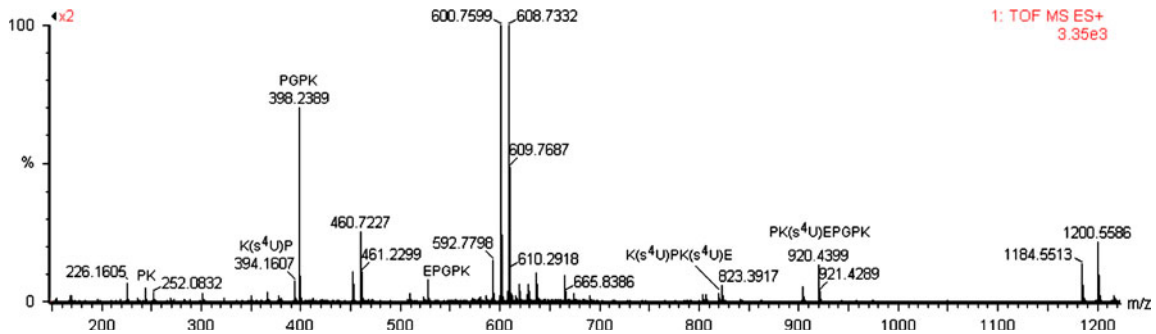


Fig. 4 MS spectrum of peptide K(s^{4}U)PK(s^{4}U)EPGPK **1c**

at the ion multi-charged $[M + 2H]^{2+}$ and the ion molecular are found respectively at 608.7633 and 1215.5109. Some other peptide fragments (ions y) are visible at 920.4 (ion y7, PK(s^4U)EPGPK), 527.3 (ion y5, EPGPK), 398.2 (ion y4, PGPK) and 244.1 (ion y2, PK). The fragment corresponding to the lysine appears at 129.1 (ion b1) and to K(s^4U)P at 394.1 (ion b2).

Conclusions

In conclusion, we have synthesized three new KPGEPGPK analogues in which the probe photochemical selectively activable 4-thiouracil (s^4U) is incorporated in position 1 and/or 3 by Fmoc chemistry synthesis with a total yield superior at 25%. These analogues could be used to identify the putative new receptor for type III collagen named Type III Collagen Binding Protein (TIIICBP).

Acknowledgment Viviane Silva Pires was the recipient of a grant from the CNPq (Brazil).

Conflict of interest The authors have no competing interests that might be perceived to influence the results and/or discussion reported in this paper.

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