PAPER

Synthesis of [18F]4-(4-fluorophenyl)-1,2,4-triazole-3,5-dione: an agent for specific radiolabelling of tyrosine

Flagothier Jessica,*[ab] Warnier Corentin,[b] Dammicco Sylvestre,[ab] Lemaire Christian[b] and Luxen André[ab]

We developed a new [18F] prosthetic group, the [18F]4-(4-fluorophenyl)-1,2,4-triazole-3,5-dione ([18F]F-PTAD), used for its specific ligation with tyrosine-containing peptides or protein in order to develop a new and versatile radiolabelling technique that could provide a useful tool for new developments in PET imaging.

Introduction

Peptides and proteins are largely used as radiopharmaceuticals for positron emission tomography (PET) studies. The most frequently used radionuclides for PET provide a wide range of physical half-lives that are compatible with the peptide or protein biological half-lives. They can be classified according to their mode of labelling. The peptide is covalently coupled, often *via* a spacer, to a chelator (*e.g.* DOTA, NOTA) which can complex radiometals as gallium-68, copper-64, yttrium-86[1,2] or carry a prosthetic group that can be labelled with iodine-124 or fluorine-18 ions.[3–5] The introduction of fluorine-18 requires harsh conditions (high temperature and strong base) that are not compatible with direct labelling of biomolecules. Therefore, two strategies are usually explored.

The first one is the incorporation of a linker onto the amino acid. The peptide is coupled *via* the linker to the [18F] prosthetic group. For instance, the coupling can be realized *via* Huisgen cycloaddition between [18F]fluoroethylazide[6] or 1-(azidomethyl)-4-[18F]-fluorobenzene[7] and a alkyne or azide-bearing peptide or protein, *via* photoclick to avoid the use of copper,[8] *via* oxime formation between [18F]fluorobenzaldehyde and modified peptides[9] or *via* the tetrazine *trans*-cyclooctene reaction.[10]

The second strategy is the [18F] prosthetic group ligation directly on naturally available function of amino acid side chains of peptides or proteins. *E.g.*: [18F]SFB,[11] [18F]FBEM[12,13] can be linked under mild conditions on –NH₂, –SH group of the peptide.
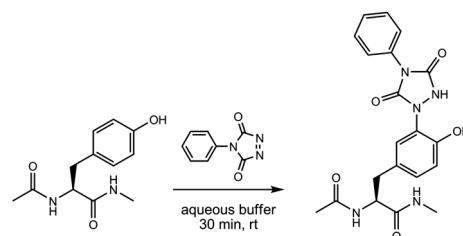
Lysine and cysteine are the most commonly functionalized amino acids: however, the high abundance of lysine makes specific modification difficult and cysteine is most often found in disulfide linked pairs in protein natural environment.

Much recent research developments have focused on the modification of tyrosine and tryptophan. These residues are relatively rare on protein surface, and offer opportunities for controlled single-site modification.

We hereby focus on the coupling between a new prosthetic group and a tyrosine-containing peptide. In literature, these reactions often use transition-metal-mediated processes.[14–17] However, transition metal mediated reactions are to be avoided as much as possible in radiochemistry. The transition metals are indeed often toxic and must be removed efficiently from the sample prior to injection, which complicates and lengthens both the synthesis and quality control of the radiotracer. Francis and co-workers have also explored the labelling of tyrosine residues through a three-component Mannich-type reaction with aldehydes and anilines.[18–20] But these reactions are not transposable in [18F] radiochemistry due to the long synthesis time (20–24 h).

Recently, Ban and co-workers have reported a tyrosine bioconjugation through ene-type reactions[21,22] (Scheme 1).

In this reaction, the cyclic diazodicarboxamide PTAD, an electrophilic compound, reacts selectively on the *o*-position of the phenol side chain of tyrosine in mild aqueous conditions (aqueous buffer). They showed that histidine, serine and cysteine were not modified by PTAD and determined that tryptophan and lysine did not interfere with the modification of tyrosine. That indicates that this reagent exhibits a high degree

[a]*Département de chimie, Université de Liège, 3 allée du 6 aout, B-4000 Liège, Belgium. E-mail: jflagothier@ulg.ac.be; Fax: +32 4 3662946; Tel: +32 4 3662334*

[b]*Centre de Recherches du Cyclotron, Université de Liège, 8 allée du 6 aout, B-4000 Liège, Belgium. E-mail: aluxen@ulg.ac.be; Fax: +32 4 3662946; Tel: +32 4 3663687*

Scheme 1 Tyrosine bioconjugation through ene-type reaction developed by Ban and co-workers.

of chemoselectivity. They also demonstrated that the 1,2,4-triazolidine-3,5-dione linkage is hydrolytically and thermally stable, a great advantage for future *in vivo* injections.

Due to these positive properties, this technique was chosen to link a [18F] prosthetic group to the tyrosine included in a peptide. We herein present the synthesis of [18F]4-(4-fluorophenyl)-1,2,4-triazole-3,5-dione [18F]F-PTAD and the preliminary results for the coupling with tyrosine.

Results and discussion

We initially focused on the direct introduction of fluorine-18 on the cyclic diazodicarboxamide **1**. Compound **1** can be easily prepared in three steps with 4-nitrobenzylisocyanate as starting material[21,23,24] but cannot be isolated due to its high instability.[25] The second approach to obtain compound **4** was the labelling of 4-(4-nitrophenyl)-1,2,4-triazolidine-3,5-dione **2** followed by the triazolidine ring oxidation step (Scheme 2).

Unfortunately, all the attempts to obtain this compound [18F]**3** (Kryptofix/K2CO3, organic bases method[26] or "C14" method[27]) at temperatures between 90 °C and 180 °C were unsuccessful. The labelling conditions are too harsh and only 18F fluoride and decomposition products of 4-(4-nitrophenyl)-1,2,4-triazolidine-3,5-dione precursor were recovered.

A second possibility was the addition of an aliphatic chain containing a tosylate leaving group on the PTAD compound (Scheme 3). Because of the poor results obtained during this aliphatic precursor synthesis including high instability of the triazolidine ring (data not shown), the strategy was changed and the fluorine-18 was introduced at the beginning of the synthesis.

Precursor **5** was synthesized according to the literature using 4-nitroaniline as starting material[28] and involved two reaction steps: dimethylation of 4-nitroaniline with iodomethane and trimethylammonium triflate formation with methyltrifluoromethanesulfonate. Precursor **5** was obtained with a global yield of 28%.

During the radiosynthesis of compound [18F]**10** (Scheme 4), the identification of [18F] compounds **6–10** was based on radio-TLC and radio-HPLC analyses showing the same eluting factor or retention time as the corresponding cold references compounds.

Compound **5** was labelled in DMSO at 100 °C in 10 minutes with standard KF[18F]-K222/potassium carbonate conditions to afford [18F]**6** with $91 \pm 4\%$ of radiochemical yield (decay corrected). [18F]**6** was diluted in water and washed on a C18 Sep-Pak cartridge to eliminate the remaining precursor and other
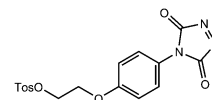

Scheme 3 Aliphatic precursor derived from PTAD compound.

impurities. This is why precursor **5** was preferred to the 1,4-dinitrobenzene which is hardly separated from 4-fluoronitrobenzene. Reduction of the nitro group with sodium borohydride in the presence of palladium on activated carbon in methanol gave compound [18F]**7** with a radiochemical yield (DC) of $80 \pm 6\%$.[28]

This reduction method is well suited because Pd/C can be easily filtered through a glass fibre filter (Millex) before the trapping onto a *t*C18 environmental Sep-Pak® cartridge. Since the next step requires a dry reaction medium, the cartridge is dried under a stream of nitrogen. [18F]**7** is then eluted with dry dichloromethane and coupled with ethyl carbazate in the presence of triphosgene and triethylamine.[29,30] This method was preferred to the one proposed by Mallakpour *et al.*,[31] which is more longer, inapplicable to the fluorine-18 chemistry, and affords lower yield. Directly after the evaporation of dichloromethane, cyclisation of semicarbazide [18F]**8** was realized as described by Cookson *et al.*[24] in basic aqueous medium and [18F]**9** was obtained in acetonitrile after its purification on a *t*C18+ short Sep-Pak® cartridge. The radiochemical yield (DC) for the coupling and cyclisation steps is $65 \pm 5\%$. The last step is the triazolidine ring oxidation with *N*-bromosuccinimide and pyridine in acetonitrile. The global radiochemical yield for the synthesis of [18F]4-(4-fluorophenyl)-1,2,4-triazole-3,5-dione is 50% (DC).

Because of the high instability of the F-PTAD compound, the crude solution of [18F]**10** was directly used, without purification, in tyrosine ligation reaction. We chose *N*-acyl tyrosine methylamide as a model for the preliminary studies of the ligation of [18F]F-PTAD with a tyrosine, as described by Ban and co-workers[21] (Scheme 5).

[18F]**11** was obtained after the coupling between [18F]**10** and *N*-acyl tyrorine methylamide in PBS/CH3CN (1/1, v/v) solution with $65 \pm 5\%$ yield. When the [18F]F-PTAD was added continuously over a period of 2 minutes, instead of a single addition, the reaction yield increased significantly from 40% to 70%. In some cases, when the tyrosine of the target compound or protein are
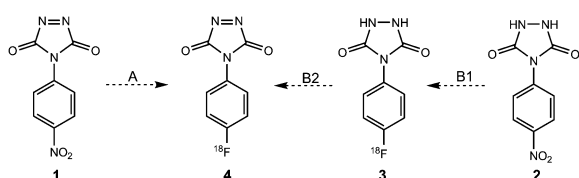
Scheme 2 (A) Radiolabelling not tested due to the high instability of compound **1**. (B) Radiolabelling followed by the triazolidine ring oxidation.
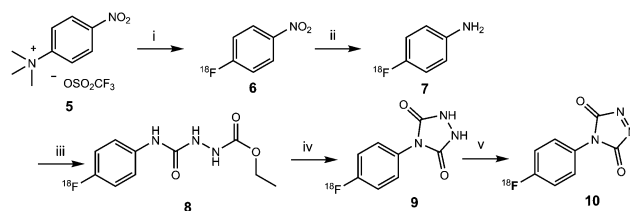
Scheme 4 Preparation of [18F]4-(4-fluorophenyl)-1,2,4-triazole-3,5-dione and coupling with *N*-acyl tyrosine methylamide. (i) KF[18F]-K222/K2CO3, DMSO, 10 min, 100 °C, 94%; (ii) NaBH4, Pd/C, MeOH, 5 min, rt, 88%; (iii) triphosgene, rt, NEt3, 5 min, 0 °C, ethylcarbazate, 10 min, 80%; (iv) 4 M KOH, 5 min, rt, 85%; (v) NBS, pyridine, CH3CN.
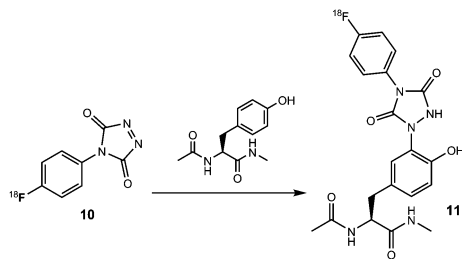
Scheme 5 Ligation of [¹⁸F]F-PTAD and *N*-acyl tyrosine methylamide: pH = 7 phosphate buffer (PBS), CH₃CN, 5 min, rt, 65%.

less accessible, the isocyanate decomposition products of PTAD compounds may be formed and urea formation at lysines is likely.[22,32] This problem is solved by using Tris buffer. In our case, the use of Tris buffer in the [¹⁸F]F-PTAD–*N*-acyl tyrosine methylamide ligation instead of PBS provides the desired tyrosine ligation product with the same yield. The crude reaction mixture was diluted with water and injected onto a semipreparative HPLC to obtain pure [¹⁸F]**11**. The global radiochemical yield (synthesis of [¹⁸F]F-PTAD, ligation with *N*-acyl tyrosine methylamide and semipreparative HPLC purification) is 20% (DC) and the synthesis lasts 2 h 15 min.

Conclusion

We synthesized a new [¹⁸F] prosthetic group, the [¹⁸F]4-(4-fluorophenyl)-1,2,4-triazole-3,5-dione ([¹⁸F]F-PTAD) with good yield and proved that this [¹⁸F]F-PTAD can be efficiently coupled with a tyrosine, the *N*-acyl tyrosine methylamide. This radiosynthesis was designed to be implemented on an automatic synthesizer. Its automation is in progress in our laboratory on a Fastlab® synthesizer from GE Healthcare.

Experimental procedure

General procedure

Reagents and solvents were purchased at the highest commercial quality and used without further purification, unless otherwise stated. NMR spectra were recorded on Bruker Avance DRX-400 instruments (¹H at 400 MHz and ¹³C at 100 MHz), ¹H and ¹³C spectra were referenced to TMS using the ¹³C or residual proton signals of the deuterated solvents as internal standards. The following abbreviations were used to designate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. All the values are expressed in part per million (ppm). TLC analyses were performed on Macherey-Nagel Polygram SIL G/UV254 plates using UV light as visualizing agent. A Bioscan TLC scanner model AR2000 was used for analysis of the ¹⁸F labelled compounds. HPLC analyses were run on a Waters system (616 pump, a manual Rheodyne injector, 996 PDA detector and NaI(Tl) scintillation detector from Eberline) controlled by Empower® software. Analytical HPLC Analyses were performed on an Xbridge® column C18 (4.6 × 150 mm; 5 μm) with a flow rate of 1 mL min⁻¹. During the radiochemical synthesis, analytical HPLC analyses were performed using the following conditions (grad1): CH₃CN/H₂O,

linear 5 min from 5/95 to 15/85, linear 15 min from 15/85 to 50/50 and the washout linear 10 min from 50/50 to 90/10. Semipreparative HPLC analyses were performed on an Xbridge® column C18 (10 × 250 mm; 5 μm) using the following conditions (grad 2): CH₃CN/H₂O, linear 30 min from 5/95 to 25/75 and linear 5 min from 25/75 to 50/50 with a flow rate of 5 mL min⁻¹.

Chemistry

p-**Fluoro-4-phenyl-1-carbethoxysemicarbazide.** This compound was synthesized according to the literature.[23]

4-(4-Fluorophenyl)-1,2,4-triazolidine-3,5-dione. This compound was synthesized according to the literature.[24]

4-(4-Fluorophenyl)-3*H*-1,2,4-triazole-3,5(4*H*)-dione. This compound was synthesized according to the literature.[21] The red solution should be used for bioconjugation without isolation.

(*S*)-2-Acetamido-3-(4-hydroxy-3-(4-(4-fluorophenyl)-3,5-dioxo-1,2,4-triazolidin-1-yl)phenyl)-*N*-methylpropanamide. This compound was synthesized according to the literature.[21]

RMN ¹H(400 MHz, DMSO-d⁶): 1.77 (s, 3H), 2.53 (d, 3H, *J* = 4 Hz), 2.61–2.66 (dd, 1H, *J* = 8 Hz, *J* = 12 Hz), 2.83–2.87 (dd, 1H, *J* = 4 Hz, *J* = 12 Hz), 4.28–4.34 (m, 1H, *J* = 4 Hz, *J* = 4 Hz, *J* = 8 Hz), 6.69 (d, 1H, *J* = 8 Hz), 6.84 (d, 1H, *J* = 8 Hz), 7.26 (t, 2H, *J* = 8 Hz), 7.58 (dd, 2H, *J* = 4 Hz, *J* = 8 Hz), 7.70 (s, 1H), 7.98 (d, 1H(NH), *J* = 4 Hz), 8.16 (d,1H(NH), *J* = 4 Hz).

RMN ¹³C (100 MHz, DMSO-d⁶): 22.7, 25.5, 37.3, 54.6, 115.1 (d, *J* = 23 Hz), 117.2, 121.6, 125.1, 127.5 (d, *J* = 9 Hz), 128.2, 130.6, 149.8, 153.0, 159.7 (d, *J* = 236 Hz), 162.2, 166.2, 169.1, 171.7.

N,N-Dimethyl-4-nitroaniline. This compound was synthesized according to the literature using 4-nitroaniline as starting material.[28] The compound was purified by recrystallization and an orange solid was obtained with a yield of 70%.

RMN ¹H(400 MHz, CDCl₃): 3.11 (s, 6H), 6.60 (d, 2H, *J* = 8 Hz), 8.11 (d, 2H, *J* = 8 Hz).

RMN ¹³C (100 MHz, CDCl₃): 40.3, 110.2, 126.1, 136.9, 154.9.

N,N,N-Trimethyl-4-nitrobenzenammonium triflate. This compound was synthesized according to the literature using *N*,*N*-dimethyl-4-nitroaniline as starting material.[28] The product was obtained as a pale yellow solid with a yield of 47%.

RMN ¹H(400 MHz, CO(CD₃)₂): 4.03 (s, 9H), 8.52 (m, 4H).

RMN ¹³C (100 MHz, CO(CD₃)₂): 57.1, 121.3 (q, *J* = 320 Hz), 122.8, 125.4, 149.1, 151.9.

Radiochemistry

No-carrier-added [¹⁸F] fluoride was obtained by proton bombardment of an [¹⁸O]-enriched water target *via* the ¹⁸O(p,n)¹⁸F reaction. The activity was trapping by passing the target water through a Sep-Pak light QMA cartridges (Waters). The fluoride ions on the cartridge were eluted by 700 μL of 50/50 CH₃CN/H₂O solution of K₂CO₃ (8 mg) and Kryptofix 222 (26 mg) into a heated conical glass vial (120 °C). This eluate was brought to dryness by azeotropic distillation after three additions of acetonitrile (150 μL) under a stream of nitrogen gas to give the no-carrier-added K[¹⁸F]-K₂₂₂ complex. A solution of 10 mg of *N*,*N*,*N*-trimethyl-4-nitrobenzenammonium triflate in 1 mL of

DMSO was added to the dried residue and the mixture was heated at 100 °C during 8 minutes. Labelling efficiency was checked by radio-TLC (silica gel, ethyl acetate/hexane 1/1 v/v), R_f values: [18F]fluoride = 0, [18F]4-fluoronitrobenzene = 0.8. HPLC analysis (grad1): t_R = 22 min. TLC radiochemical conversion = 91 ± 4% ($n > 10$).

The reaction medium was poured into water (10 mL) and trapped onto a previously activated with acetonitrile (5 mL) and washed with water (5 mL) C18 environmental Sep-Pak® cartridge (Waters). The [18F] 4-fluoronitrobenzene was rinsed with 10 mL of water in order to eliminate the starting precursor and was eluted with 2 mL of methanol in a vial containing 3 mg of Pd/C and 25 mg of NaBH₄. The mixture was stirred 5 minutes at room temperature. The reduction efficiency was checked by radio-TLC (silica gel, ethyl acetate/hexane 1/1 v/v), R_f values: [18F]4-fluoroaniline = 0.4. HPLC analysis (grad1): t_R = 14 min. Radiochemical yield (decay corrected) = 80 ± 6% ($n > 10$). The methanolic solution was poured into water (10 mL) and passed through a glass fiber filter (Millex) and trapped onto a tC18 environmental Sep-Pak® cartridge (Waters) previously activated with acetonitrile (5 mL) and washed with water (5 mL). The cartridge was flushed by a stream of nitrogen during 5 minutes and then eluted with 2.5 mL of dry dichloromethane in a vial containing 10 mg of triphosgene. The vial was cooled at 0 °C and 20 μL of dry triethylamine was added. The mixture was stirred for 5 min before the addition of 40 mg of ethylcarbazate. The solution was stirred 10 minutes at 0 °C to give [18F]p-fluoro-4-phenyl-1-carbethoxysemicarbazide. The reaction efficiency was checked by radio-TLC (silica gel, ethyl acetate/hexane 1/1 v/v), R_f values: [18F]p-fluoro-4-phenyl-1-carbethoxysemicarbazide = 0.1. HPLC analysis (grad1): t_R = 15 min. HPLC radiochemical conversion: 80 ± 10% ($n = 9$). Dichloromethane was evaporated and 0.5 mL of 4 M KOH were added, the mixture was stirred for 5 minutes and finally quenched by 0.5 mL of HCl 6 M. The cyclisation reaction efficiency was checked by radio-TLC (silica gel, ethyl acetate/hexane 1/1 v/v), R_f values: [18F]4-(4-fluorophenyl)-1,2,4-triazolidine-3,5-dione = 0.02. HPLC analysis (grad1): t_R = 4 min. Radiochemical yield DC for coupling step and cyclisation step = 65 ± 5% ($n = 7$).

The reaction medium was then poured into water (10 mL), trapped onto a tC18 plus short Sep-Pak® cartridge (Waters) previously activated with acetonitrile (5 mL) and washed with water (5 mL) and eluted with 1 mL of acetonitrile. 100 μL of pyridine solution (0.3 M in CH₃CN) was added to the previous solution followed by the addition of 100 μL of N-bromosuccinimide solution (0.3 M in CH₃CN). This mixture was directly added to 1 mL of N-acyl tyrosine methylamide solution (1 mg in 0.9 mL of PBS 100 mM pH = 7 (or Tris buffer 100 mM pH = 7)) during a period of 2 minutes. The solution was stirred at room temperature during 5 min. The coupling efficiency was checked by radio-TLC (silica gel, ethyl acetate/hexane 1/1 v/v), R_f values: [18F]final compound = 0.20, and by analytical HPLC (grad 1): t_R = 9 min. Radiochemical purity of [18F]11 was 65 ± 5% ($n = 5$). The radiolabelled compound was purified by semipreparative HPLC (grad 2): t_R = 12 min. The purified sample was analyzed by analytical HPLC to confirm his chemical and radiochemical purity. The total decay corrected radiochemical yield was 25%.

Acknowledgements

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Synthesis of two new alkyne-bearing linkers used for the preparation of siRNA for labeling by click chemistry with fluorine-18

Jessica Flagothier [a,b,*], Geoffroy Kaisin [b], Frederic Mercier [b], David Thonon [b], Nathalie Teller [c], Johan Wouters [d], André Luxen [b]

[a] Department of Chemistry, University of Liege (ULg), 13 allée du 6 aout, B-4000 Liege (Sart-tilman), Belgium
[b] Cyclotron Research Center, University of Liège, 8 allée du 6 aout, Bb-4000 Liège (Sart-tilman), Belgium
[c] EUROGENTEC S.A., building 2, Liège Science Park, 5 Rue Bois Saint-Jean, B-4102 Seraing, Belgium
[d] Department of Chemistry, University of Namur (FUNDP), 61 rue de Bruxelles, B-5000 Namur, Belgium

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ABSTRACT

Oligonucleotides (ONs) and more particularly siRNAs are promising drugs but their pharmacokinetics and biodistribution are widely unknown. Positron Emission Tomography (PET) using fluorine-18 is a suitable technique to quantify these biological processes. Click chemistry (Huisgen cycloaddition) is the current method for labeling siRNA. In order to study the influence of a linker bearing by [18F] labeled ONs, on the in vivo pharmacokinetic and metabolism, we have developed two modified ONs by two new linkers. Here we report the synthesis of two alkyne-bearing linkers, the incorporation onto a ONs and the conjugation by click chemistry with a [18F] prosthetic group.

1. Introduction

Oligonucleotides (ONs), especially small interfering RNA (siRNA), may be useful in the development of new drugs due to their fundamental cellular mechanism for silencing gene expression (RNA interference (RNAi)). RNAi is a process by which siRNAs direct the degradation of target messenger RNA (mRNA) and induce specific posttranscriptional gene silencing (Fire et al., 1998; Tavitian, 2003). The main obstacle to the development of new siRNA-drugs is the lack of knowledge regarding their pharmacokinetics and biodistribution.

Positron Emission Tomography (PET) is a sensitive molecular and functional imaging technique that permits repeated, non-invasive, assessment and quantification of specific biological and pharmacological processes in humans or animals (Ametamey et al., 2008). Among a number of positron-emitting nuclides, fluorine-18 is often the radionuclide of choice for labeling biomolecules due to its favorable physical and nuclear properties $(t_{1/2}(^{18}F)=109.7$ min) (Cai et al., 2008).

The challenge for radiochemists is to easily introduce this short half-life isotope onto oligonucleotides or, more generally, biomolecules. Direct incorporation of fluorine-18 at high specific radioactivity onto biomolecules is hampered by the harsh reaction conditions occurring during labeling reactions (Schulz et al., 2011). In order to circumvent this obstacle, the method of choice for highly efficient fluorine-18-labeling of oligonucleotide-type macromolecules is currently the conjugation of a prosthetic group carrying the radioisotope with a reactive function of the macromolecule.

Several pathways for the introduction of a prosthectic group for labeling ONs with fluorine-18 have been developed: via acylation with succinimido 4-[18F]fluorobenzoate (Hedberg and Langstrom, 1998), alkylation (de Vries et al., 2003) with, e.g., N-(4-fluorobenzyl)-2-bromoacetamide (FBBA) (Dolle et al., 1997; Kuhnast et al., 2000, 2003) and 2-bromo-N-[3-(2[18F]fluoropyridin-3-yloxy)propyl]acetamide (FPyBrA) (Kuhnast et al., 2004; Viel et al., 2007), photochemical conjugation with 3-azido-5-nitrobenzyl-[18F]fluoride (Lange et al., 2002) and thiourea formation with 4-([18F]fluoromethyl)phenyl isothiocyanate (Hedberg and Langstrom, 1997). The introduction of [18F]FBBA or [18F]FPyBrA onto the ONs by nucleophilic substitution has suboptimal global yields (17% and 5%, respectively) and requires a long synthesis time (150 min and 85 min, respectively) (Dolle et al., 1997; Kuhnast et al., 2004, 2000).

A method to introduce a prosthetic group while keeping mild conditions required for ONs stability is the click chemistry (Mercier et al., 2011; Shiraishi et al., 2011) (Fig. 1). In our previous studies, the connection between the [18F] prosthetic group and

* Corresponding author. Tel.: +32 4 3662322; fax: +32 4 3662946.
  E-mail address: jflagothier@ulg.ac.be (J. Flagothier).

the siRNA has been realized by means of a diol carboxylic linker derivated from hydroxybutyrolactone (Mercier et al., 2011).

In order to study the influence of the linker, bearing by labeled ONs, on the in vivo pharmacokinetic and metabolism, we have developed two modified ONs by two new linkers.

These two new linkers contain, as described by Mercier et al., a primary alcohol with a dimethoxytrityl group to start ON sequence, a secondary alcohol with a functional group allowing connection between the linker and the support solid and an amino group to connect the alkyne arm. The first linker synthesized is an *N*-heterocycle derivative of proline. This structure mimic a sugar associated with the oligonucleotide sequence, thus providing a well-defined and known stereochemistry supporting optimal in vivo conditions and minimizing or avoiding metabolic degradation (Ceulemans et al., 1997; Efimov et al., 2010). The second linker synthesized is derived from threonine. Much more similar to the linker synthesized by Mercier et al., this molecule is also a γ-diol, the differences are the amide connection and the presence of a second asymmetric center.

Fig. 1. Coupling between prosthetic groups and modified ONs with linkers using Click Chemistry.

Some studies was realized to demonstrate the best in vivo stability of a linker at the 3′-end of oligonucleotides compared to the stability of this linker at the 5′-end of oligonucleotides (Temsamani et al., 1993). We have thus decided to study in vivo the influence of the structure of the linker attached at 3′-end of the oligonucleotide sequence.

The syntheses of two linkers (**10** and **21**) are proposed. Alkyne-linker **10** was attached at the amino group of the controlled pore glass (CPG) support. The sense-strand was synthesized from this solid support and annealed with the antisense-strand. The double-stranded siRNA was thus synthesized with the alkyne-linker at the 3′ extremity of the sense-strand siRNA **23** and engaged in the 1,3-dipolar Huisgen cycloaddition with the ^{18}F or ^{19}F synthon 1-(azidomethyl)-4-fluorobenzene **24** to afford the ^{18}F or ^{19}F siRNA **25** which can be used for the cold reference and for in vivo PET imaging studies, respectively.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of linker 10

Trans-4-hydroxy-L-proline **1**, commercially available, was considered as the starting material. This linker will mimic a sugar because proline and ON sugars have a similar structure. As described above, this provides a favorable linker.

The first strategy for the synthesis of this linker was based on a protection-deprotection system with diverse protecting groups to avoid side reactions. (Scheme 1) Trans-4-hydroxy-L-proline was converted into an ester by conventional methods using thionyl chloride and methanol esterification (Westwood and Walker, 1998). After Fmoc protection (Booth et al., 1998) (60% yield), compound **3** was reduced by in situ generation of borane with NaBH$_4$/I$_2$(Lutsenko et al., 2003) with 45% yield. The two hydroxyl groups were protected by triisopropylsilyl chloride to afford the
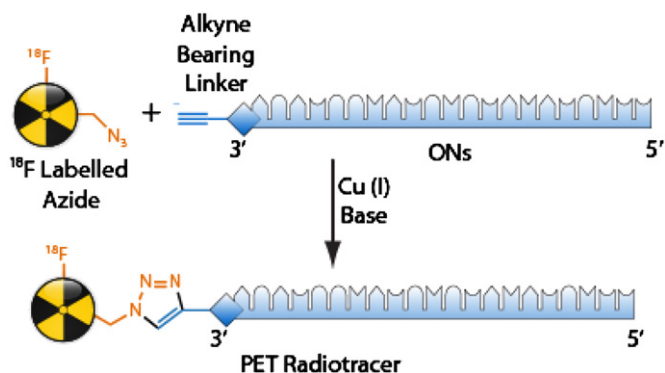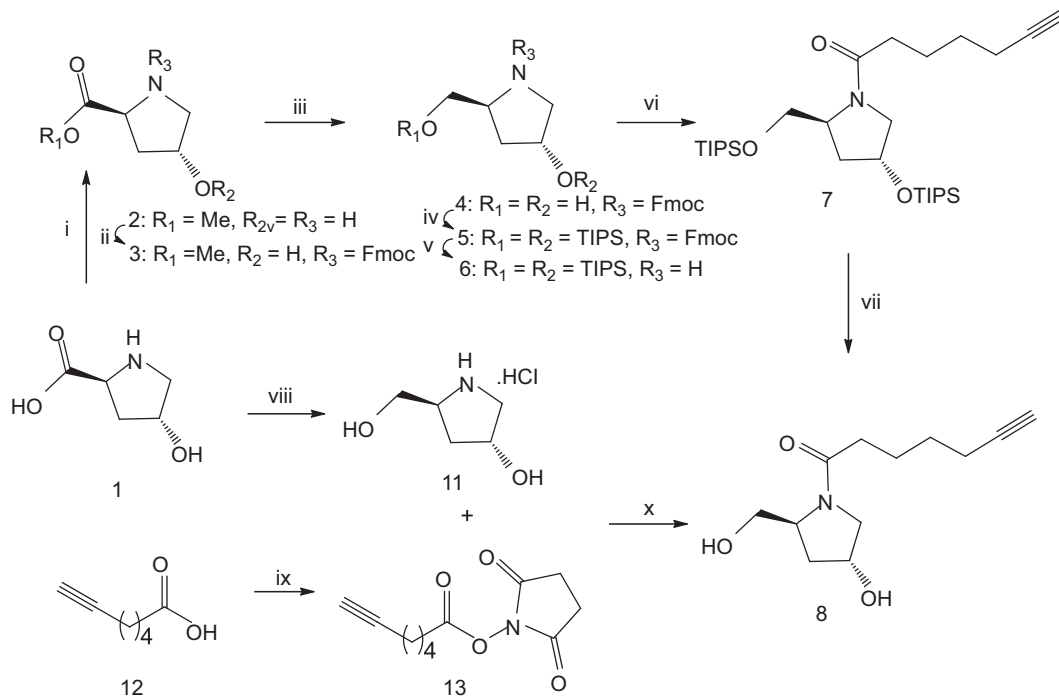

Scheme 1. Reaction scheme for the preparation of the (2S,4R)–*N*-(6-heptynoyl)-4-hydroxy prolinol **8**. Reagents and conditions: (i) SOCl$_2$, dry MeOH, rt; (ii) FmocOSu, Na$_2$CO$_3$, dioxane, 24 h; (iii) NaBH$_4$, I$_2$, reflux, 24 h; (iv) TIPSCl, imH, CH$_2$Cl$_2$, 0 °C, 24 h; (v) piperidine 20% THF; (vi) 6-heptynoic acid **12**, DIEA, HBTU, CH$_2$Cl$_2$, DMF, rt, 12 h; (vii) HCl/MeOH 1.7 M, rt, 2 h; (viii) HMDS, TMSCl, CH$_3$CN, reflux, 9 h, BH$_3$.DMS, 1,4-dioxane, reflux 10 h, H$_2$O/HCl, reflux, 1 h; (ix) TSTU, NEt$_3$, CH$_2$Cl$_2$, CH$_3$CN, rt, 30 min; (x) NEt$_3$, dry MeOH, rt, 2 h.

fully protected derivative **5** (Kamal et al., 2004) with 90% yield. Stirring of this Fmoc-protected compound in a solution of 20% piperidine in THF (Nuti et al., 2007) afforded the corresponding Fmoc-deprotected compound **6**. This compound was directly coupled with 6-heptynoic acid **12** leading to compound **7** with 61% yield. The best yield of coupling reactions was obtained in a mixture of dimethyformamide and dichloromethane in the presence of diisopropylethylamine (DIEA) and HBTU (Tedeschi et al., 2002). Triisopropylsilyl groups, necessary to avoid esterification during the coupling step, were removed with a solution of 1.5 M HCl in methanol to afford **8** (35% yield) as a crystalline solid. X-ray
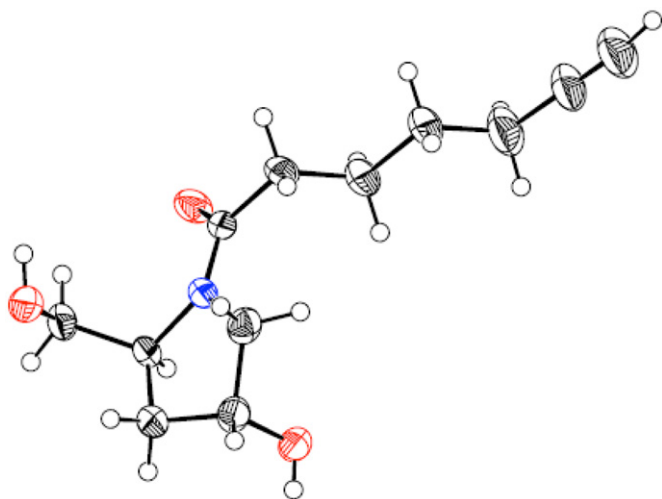

Fig. 2. Crystal data (ORTEP) for **8**.

crystallographic confirmed the trans-structure as presented in Fig. 2.

Due to the high number of steps and the low yield of some steps, an alternative chemical synthesis was developed: Scheme 1 was elaborated upon to improve the yield of the steps between compound **1** and **8**. This new synthesis method is presented in Scheme 1.

This modified synthesis also started with commercially available *trans*-4-hydroxyproline **1**, which directly underwent a quantitative reduction to *trans*-4-hydroxyprolinol **11** using a dimethylsulfide complex of borane (Folkersen et al., 1999; Godskesen et al., 1996). Other classical carboxylic acid reduction methods, i.e., $LiBH_4$ reduction or $LiAlH_4$ reduction of the corresponding methyl ester, were investigated but did not provide satisfactory yields. The coupling reaction between *trans*-4-hydroxyprolinol **11** and 6-heptynoic acid **12** was next examined. Classical peptide coupling reagents are known to be inefficient in the presence of free alcohol moieties. Consequently, four activated forms of 6-heptynoic acid **12** were synthesized, succinimidyl ester **13** (Bannwarth and Knorr, 1991), *N*-acylthiazolidinethione **14** (Nagao et al., 1980), pentafluorophenyl ester **15** (Christensen, 2001; Wellendorph et al., 2003) and *N*-acylbenzotriazole **16** (Katritzky et al., 1992) (Fig. 3).

These activating agents have reacted with **11** to afford compound **8** with different yields. The results are presented in Table 1.

These activating agents appeared to tolerate free hydroxyl groups and showed a high *N*-acylation versus *O*-acylation selectivity. After analysis of these results, it was decided to use TSTU as coupling agent (Bannwarth and Knorr, 1991). Using this method, compound **8** was synthesized with 60% yield (Table 1, Entry 1) (Scheme 1), with a clear improvement compared to the first method (Scheme 1) (5% yield).

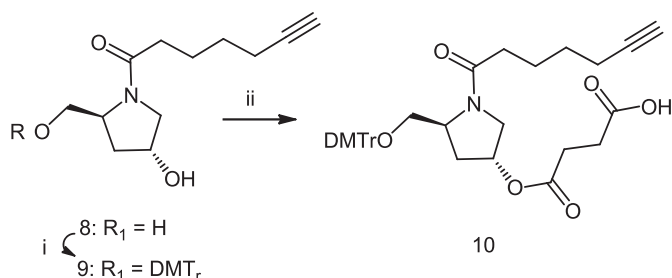| Activating agent		Activated acid[a]		Yield
TSTU			**13**	91%
TTH			**14**	38%
Pentafluorophenol			**15**	31%
1*H*-benzotriazol			**16**	33%

[a]Reaction of activating agent with 6-heptynoic acid. The couplings with TTH, pentafluorophenol and 1H-benzotriazole need *in situ* acid chloride formation with $SOCl_2$.

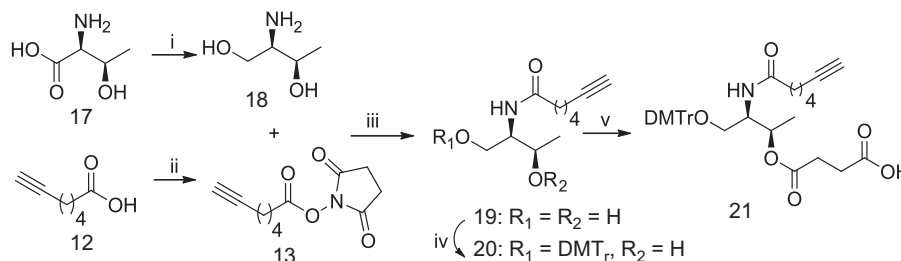**Fig. 3.** Activating agents and activated acids.

Next, the primary hydroxyl group of **8** was selectively pro-tected using the DMTr ether (Scheme 2). In spite of the use of inert atmosphere and freshly purified reagent, the best yield obtained was 15%. The remaining secondary hydroxyl group in **9** was esterified by treatment with succinic anhydride in acet-onitrile (26% yield) to afford the final linker **10**.

Table 1
Coupling conditions for activating agents **13**, **14**, **15** and **16** with (2S,4R)-4-hydroxyprolinol hydrochloride **11** to afford compound **8**.
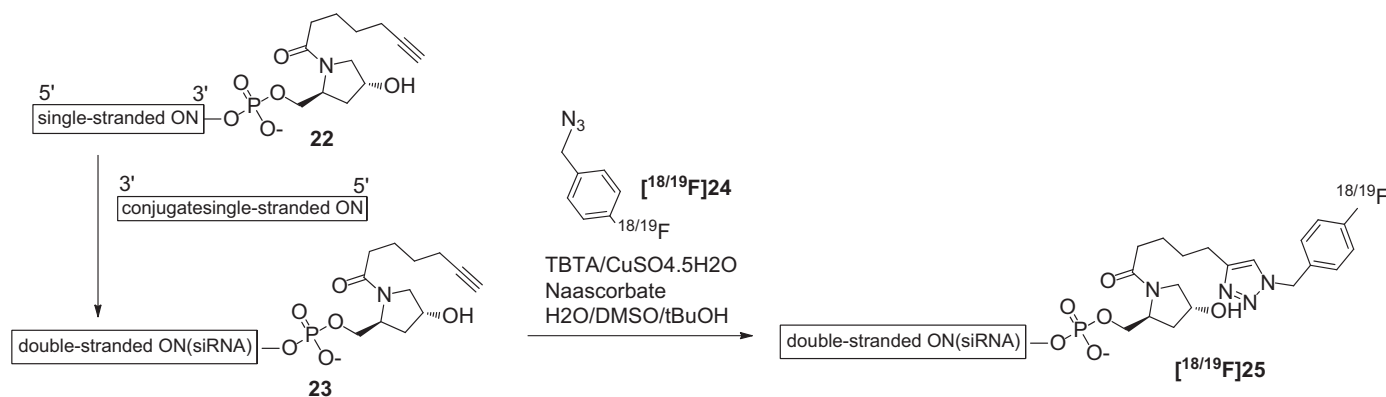
Entry	Activated agent	Procedure	Solvent	Eq. activated agent	Eq. NEt$_3$	Time	Yield
1	**13**	B	MeOH	0.5	3	1 h30	60%
2	**14**	C	THF	0.9	5	1 h30	34%
3	**15**	D	CH$_2$Cl$_2$	1.1	2.1	20 h	13.5%
4	**16**	E	MeOH	1	2.1	24 h	15%

Scheme 2. Reaction scheme for the preparation of the compound 4-((3S,5R)-5-((dimethoxytrityloxy)methyl)-1-(6-heptynoyl)-3-pyrrolidinyloxy)-4-oxobutanoic acid **10**. Reagents and conditions: (i) DMTrCl, DMAP, NEt$_3$, CH$_3$CN, rt, 12 h; (ii) Succinic acid, DMAP, NEt$_3$, CH$_3$CN, rt 12 h.

2.1.2. Synthesis of linker 21

Threoninol as starting material was chosen to afford linker **21**. This compound meets all the requirements needed, described by Mercier et al., primary alcohol, secondary alcohol and presents on the second asymmetric center an amino group which is easily functionalisable to introduce an alkyne chain. Easily commercially available at low cost, derivated from natural threonine, enantio-merically pure, these criteria made us choose threoninol as starting material. (Scheme 3) This linker was obtained in the same conditions as previously described for compound **10** with 24% yield.

2.2. Synthesis of modified siRNA

The product **10** was used directly for immobilization on LCAA-CPG and oligonucleotide synthesis at Eurogentec (Liège, Belgium). The resin load was 33 μmol/g. The sense-strand and the anti-sense-strand of the siRNA were synthesized with modified alkyne solid support **10** and a normal solid support, respectively, and finally annealed according to Mercier et al. (2011). The following oligonucleotide sequences (synthesized by Eurogentec) were used: 5-UCACUUACAGGAUCUAUAA-3 (sense) and 5-UUAUA-GAUCCUGUAAGUGA-3 (antisense)(Garbacki et al., 2009).

2.3. Click chemistry

The modified siRNA **23** was clicked with 1-(azidomethyl)-4-fluorobenzene [^{19}F]**24** (Demko and Sharpless, 2002).

For this click reaction, azide [^{19}F]**24** and an excess of Cu(II)/TBTA ligand complex 1/1, sodium ascorbate in a H$_2$O/Me$_2$SO solution were added to the double-stranded modified siRNA **23** as described by Mercier et al. As described in literature, different solvent mixtures (H$_2$O, dioxane, Me$_2$SO, t-BuOH, etc) with an excess of sodium ascorbate and Cu(II)/TBTA complex are gener-ally used for click reaction with ONs (Berndl et al., 2009;

Scheme 3. Reaction scheme for the preparation of the compound N-(2R,3R)-1-dimethoxytrityloxy-3-succinoyl-2-butanyl-6-heptynamide **21**. Reagents and conditions: (i) HMDS, TMSCl, CH$_3$CN, reflux, 9 h, BH$_3$.DMS, 1,4-dioxane, reflux 10 h, H$_2$O/HCl, reflux, 1 h; (ii) TSTU, NEt$_3$, CH$_2$Cl$_2$, CH$_3$CN, rt, 30 min; (iii) NEt$_3$, dry MeOH, rt, 2 h; (iv) DMTrCl, pyridine, dry THF, rt, 12 h; (v) Succinic anhydride, DMAP, pyridine, 50 °C, 12 h.

Scheme 4. Synthesis of ^{18}F-labeled siRNA [^{18}F]**25** and cold reference [^{19}F]**25**.

El-Sagheer and Brown, 2009; Kiviniemi et al., 2008). After semi-preparative HPLC purification and lyophilisation, the siRNA [^{19}F]25 was obtained. (Scheme 4).

2.4. Radiochemistry

No-carrier-added fluorine-18 was obtained by proton bombardment of [^{18}O]-enriched water ($>95\%$) via the ^{18}O(p,n)^{18}F nuclear reaction.

The modified siRNA **23** was clicked with 1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{18}F]24. (Scheme 4) The radiosynthesis of this prosthetic group was fully automated on a FASTLab synthesizer from *GE HealthCare*. The ^{18}F-azide has been obtained according to Thonon et al. (2009) with a modification: the 1-(azidomethyl)-4[^{18}F]fluorobenzene [^{18}F]24 trapped on OASIS HLB LP+ was eluted with 6 mL of a solution of Me$_2$SO/diethyl ether: 0.5/5.5. After elimination of the ether by evaporation under N$_2$, 0.5 mL of 1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{18}F]24 obtained was used directly for the click reaction. The duration of 1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{18}F]24 synthesis was 45 min. This synthon was obtained with a radiochemical yield of 84% (decay corrected).

The click ligation between 1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{18}F]24 and the double-stranded siRNA **23** was also performed with a large excess of Cu(II)/TBTA complex and sodium ascorbate in a solvent mixture of H$_2$O/Me$_2$SO 19/81 (v/v) as described by Mercier et al. For the same radiochemical yield in comparaison with Mercier et al., the quantity of siRNA used in the initial steps was reduced by a factor 10. This represents an important advantage of the method considering the high cost of this molecule. The crude click product was diluted with HPLC solvent and purified by semipreparative HPLC. The fraction containing the pure [^{18}F]siRNA **25** was collected and diluted with water, trapped on a tC18 plus Sep Pak cartridge, eluted with ethanol and finally transferred onto NAP10 gel filtration. The [^{18}F]siRNA **25** (2–10 mCi) was eluted with physiological solution to obtain the pharmaceutical form. The duration of the radiosynthesis of (1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{18}F]24, click reaction, HPLC and formulation) was 120 min. Pure siRNA [^{18}F]25 was obtained with a radiochemical yield of 12% (decay corrected).

3. Conclusion

In summary, two new linkers **10** and **21** have been synthesized following well-defined aspects. Linker 10 owns a similar structure to that sugar of oligonucleotide, this is a advantage to minimize metabolic degradation (Ceulemans et al., 1997). These alkyne-bearing linkers **10**, **21** can be incorporated onto solid-phase supports of ONs. The method is universal because any sequence of oligonucleotide can be synthesized from these linkers attached to the CPG. These ONs, (single or double-stranded) functionalized by an alkyne, can be coupled with any azido-bearing prosthetic group by click reaction. The mild click chemistry conditions eliminate the risks of ON degradation. SiRNA [^{18}F]25 was obtained with this method by means of 1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{18}F]24 developed (Thonon et al., 2009) and fully automated (Mercier et al., 2011) in our laboratory. This synthon was produced in 45 min with a 84% radiochemical yield. The low siRNA concentration ($\sim 10^{-5}$ M) needed for click reaction represents an important advantage of the method considering the high cost of this molecule. The quantity of [^{18}F]siRNA obtained allows direct use (2–10 mCi), after purification and formulation, for in vivo PET imaging studies.

Some preliminary PET studies will be carried with modified siRNA [^{18}F]25.

These ONs, (single or double-stranded) functionalized by an alkyne, can be also coupled with any DOTA derivatives comprise amine-reactive functionalities. The synthesized ON–DOTA-conjugates will be labeled with ^{68}Ga (Wängler et al., 2011).

4. Material and methods

4.1. General procedures

Reagents and solvents were purchased at the highest commercial quality and used without further purification, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on Macherey-Nagel Polygram SIL G/UV254 plates using UV light as visualizing agent and an ethanolic solution of anisaldehyde, and heat as developing agents. NMR spectra were recorded on Bruker Avance DRX-400 instruments (^{1}H at 400 MHz and ^{13}C at 100 MHz) ^{1}H and ^{13}C spectra were referenced to TMS using the ^{13}C or residual proton signals of the deuterated solvents as internal standards. The following abbreviations were used to designate the multiplicities: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet. All the values are expressed in part per million (ppm). HPLC analyses were run on a Waters system (616 pump, a manual Rheodyne injector, 996 PDA detector and NaI(Tl) scintillation detector from Eberline) controlled by Empower® software. HPLC Analyses were performed on a SYMMETRY® column C18 (3.9×150 mm; 5 µm), semipreparative HPLC analyses on a X-TERRA® column C18 (7.8×300 mm; 7 µm) and preparative HPLC on NOVASEP column LC50.500VE150 with Kromasil®. For siRNA HPLC analyses were performed on a Jupiter Proteo 90 Å column from Phenomenex (150×4.6 mm) using the following conditions: A=TEAA 0.1 M (pH 7.4) in H$_2$O/CH$_3$CN: 19/1; B=MeOH; gradient elution: 100% of A for 1 min then linear 30 min from 10/0 to 2/8 (A/B), then washout 6 min at 100% B; flow rate: 1 mL/min; temperature: rt; absorbance detection at $\lambda = 254$ nm. Semipreparative HPLC analyses for siRNA were performed on a Jupiter Proteo 90 Å column from Phenomenex (250×10 mm) with the same gradient (rt, 254 nm); flow rate: 4 mL/min. Mass spectra were recorded on a Thermoquest Finnigan TSQ 7000 mass spectrometer (Thermo-ElectronCorp.) operating in full scan MS mode with an ESI+ source. Mass spectroscopy experiments of siRNA were performed on a Q-TOF Ultima Global (Micromass, now Waters, Manchester, UK) with the normal ESI source. Melting points were recorded on a Büchi Melting Point B-545 apparatus.

4.2. Chemistry

(2S,4R)-4-Hydroxy-2-(methoxycarbonyl)pyrrolidinium chloride (2)

This compound was synthesized according to the literature (Westwood and Walker, 1998).

(2S,4R)–N-(fluoren-9-ylmethoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic Acid Methyl Ester (3)

N-(Fluoren-9-ylmethoxycarbonyl)succinimide (24.2 g, 72 mmol) in dioxane (100 mL) was added to a stirred solution of **2** (13 g, 72 mmol) and 9% aqueous sodium carbonate (100 mL). Stirring at room temperature was continued overnight. The reaction mixture was diluted with water (100 mL) and washed with ethyl acetate (3×100 mL). The aqueous phase was acidified with concentrated hydrochloric acid and extracted with ethyl acetate (2×100 mL). The organic phase was dried over anhydrous MgSO$_4$, filtered and concentrated under reduced pressure. The product was purified by chromatography on silica gel with (CH$_2$Cl$_2$/MeOH 19:1, v/v) and compound **3** was obtained as a colorless foam (19.3 g, 60%). TLC (CH$_2$Cl$_2$/MeOH 19:1, v/v): $R_f = 0.33$; Analytical HPLC (CH$_3$CN/H$_2$O 6:4

v/v, 1 mL/min): t_R=2.7 min; ESI-MS (ES$^+$): m/z=368 [M+1]$^+$ (100%), m/z=390 [M+Na]$^+$ (70%), m/z=431 [M+Na+CH$_3$CN]$^+$ (40%); ^{1}H NMR (400 MHz, CDCl$_3$): 2.14 (m, 1H), 2.35 (m, 1H), 3.57 (d, J=11.8 Hz, 1H), 3.69 (dd, J=6.8 Hz, 11.8 Hz, 1H), 3.77 (s, 3H), 4.18 (t, J=6.2 Hz, 1H), 4.28 (t, J=6.8 Hz, 1H), 4.35–4.57 (m, 3H), 7.33 (m, 2H), 7.42 (m, 2H), 7.60 (m, 2H), 7.79 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$): 39.3, 47.2, 52.5, 55.3, 58.0, 67.8, 70.1, 120.0, 125.1, 127.1, 127.8, 141.3, 155.0, 173.2.

(2S,4R)–N-(fluoren-9-ylmethoxycarbonyl)-4-hydroxy prolinol (4)

A round-bottomed flask was charged with sodium borohydride (1.33 g, 34.7 mmol) and 50 mL of dry THF. The solution was stirred while compound **3** (3.2 g, 8.6 mmol) was added. A solution of iodine (3.3 g, 13 mmol) in 25 mL of THF was added dropwise to the mixture under inert atmosphere at 0 °C during a period of 30 min. The solution was allowed to warm to room temperature and the reaction mixture was brought to reflux overnight. Methanol was added dropwise to the cloudy white suspension at room temperature until dissolution of the white material. The clear solution was acidified with conc. HCl to pH 2–3. The precipitated solid was filtered off and the concentrate filtrate was purified by column chromatography on silica gel (100% ethyl acetate). Product containing fractions were combined and evaporated and co-evaporated with ethanol giving product **4** as a white solid (1.4 g, 45%). Melting point: 123.6–124.4 °C. TLC (ethyl acetate 100%): R_f=0.1; Analytical HPLC (CH$_3$CN/H$_2$O 6:4, 1 mL/min): t_R=1.8 min; ESI-MS (ES$^+$): m/z=340 [M+1]$^+$ (100%); ^{1}H NMR (400 MHz, CDCl$_3$): 1.73 (m, 1H), 2.00 (m, 1H), 3.40 (m, 1H), 3.53 (m, 1H), 3.64 (m, 1H), 3.69 (m, 1H), 4.14 (m, 1H), 4.19 (m, 1H), 4.34 (m, 1H), 4.38 (m, 2H), 7.28 (m, 2H), 7.38 (m, 2H), 7.56 (m, 2H), 7.74 (m, 2H); ^{13}C NMR (100 MHz, CDCl$_3$): 37.4, 47.2, 55.6, 59.3, 66.1, 67.7, 69.2, 120.0, 125.1, 127.1, 127. 8, 141.4, 143.9, 157.2.

(2S,4R)-(9H-fluoren-9-yl)methyl 4-((triisopropylsilyl)oxy)-2-(((triisopropylsilyl)oxy)methyl)pyrrolidine-1-carboxylate (5)

Triisopropylsilylchloride (2.34 mL, 0.011 mmol) was added to a solution of compound **4** (1.88 g, 3.6 mmol) and imidazole (0.75 g, 0.011 mmol) in dichloromethane (50 mL) and allowed to stir at 0 °C overnight. The reaction mixture was poured into water and then extracted with diethyl ether (2 × 50 mL). The combined organic phases were dried over MgSO$_4$ and the solvent was removed under vacuum. Product **5** was obtained as a yellowish oil (3.1 g, 90%) after purification by column chromatography on silica gel (hexane/ethyl acetate 19:1, v/v). TLC (Hexane/ ethyl acetate 19:1, v/v) R_f=0.18; ESI-MS (ES$^+$): m/z=430 [M-Fmoc]$^+$ (100%), m/z=652 [M+1]$^+$ (70%). ^{1}H NMR (400 MHz, CDCl$_3$): 0.9 (d, J=7.1 Hz, 36H), 1.26 (m, 6H), 2.08 (m, 1H), 2.24 (m, 1H), 3.45 (m,1H), 3.56 (m,1H), 3.65 (m,1H), 3.70 (m, 1H), 4.05 (m, 1H), 4.23 (m, 1H), 4.35 (m, 1H), 4.65 (m, 2H), 7.20 (m, 2H), 7.39 (m, 2H), 7.60 (m, 2H), 7.74 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$): 12.1, 17.9, 37.7, 47.5, 55.8, 58.5, 64.2, 67.1, 70.2, 120.1, 125.2, 127.1, 127.7, 141.4, 144.4, 155.1.

(2S,4R)-4-((triisopropylsilyl)oxy)-2-(((triisopropylsilyl)oxy)methyl) pyrrolidine (6)

Compound **5** was treated with 20% piperidine in THF at room temperature for 1 h. The solvent was evaporated and the white solid obtained was directly used in the next step. ESI-MS (ES$^+$): m/z=430 [M+1]$^+$ (100%)

1-((2S,4R)-4-((triisopropylsilyl)oxy)-2-(((triisopropylsilyl)oxy)methyl)pyrrolidin-1-yl)hept-6-yn-1-one (7)

6-heptynoic acid (0.28 mL, 2.3 mmol) was dissolved in DMF (5 mL) and added to the HBTU (1.28 g, 3.4 mmol) dissolved in CH$_2$Cl$_2$ (3 mL). DIEA (1.2 mL, 6.9 mmol) was added and the solution was allowed to stand at room temperature for the activation time (2 h). The compound **6** (2 g, 4.6 mmol) was added and the mixture was stirred overnight at room temperature.

The reaction mixture was poured into water and then extracted with ethyl acetate (2 × 100 mL). The combined organic phases were dried over Na$_2$CO$_3$. The solvent was removed under vacuum and the product was purified by column chromatography on silica gel (ethyl acetate/hexane 4:1, v/v). Product **7** was obtained as a yellowish oil (1.6 g, 61%). TLC (ethyl acetate/hexane 4:1, v/v): R_f=0.5, ESI-MS (ES$^+$): m/z=538 [M+1]$^+$ (100%), m/z=560 [M+Na]$^+$ (10%). ^{1}H NMR (400 MHz, CDCl$_3$): 1.02 (d, 36H), 1.18 (m, 6H), 1.57 (m, 2H), 1.72 (m, 2H), 1.89 (s, 1H), 1.92 (m, 1H), 2.16–2.21 (m, 5H), 3.28 (m, 1H), 3.61 (m, 2H), 4.05 (m, 1H), 4.27 (m, 1H), 4.68 (m, 1H). ^{13}C NMR: 12.0, 17.9, 18.1, 24.0, 28.3, 34.4, 37.2, 56.3, 57.8, 63.7, 67.3, 71.0, 84.3, 171.4.

(2S,4R)–N-(6-heptynoyl)-4-hydroxy prolinol (8)

Procedure A: Compound **7** (100 mg, 0.19 mmol) was dissolved in HCl/MeOH 1.7 M solution. To prepare this solution, gaseous HCl was introduced in MeOH. Gaseous HCl was prepared by the dropwise addition of H$_2$SO$_4$ on NaCl. The mixture was stirred at room temperature for 4 h. NaHCO$_3$ solid was added to the solution to neutralize acidity. The solvent was removed under vacuum and the residue was purified by column chromatography on silica gel (CH$_2$Cl$_2$/MeOH 9:1, v/v). Product **8** was obtained as a white solid (15 mg, 35%).

Procedure B: Compound **11** (695 mg, 4.5 mmol) was dissolved in dry MeOH (15 mL) and NEt$_3$ (681 mg, 6.75 mmol) and the solution was stirred for 15 min at room temperature. Compound **13** (500 mg, 2.25 mmol) was added and the mixture was left for 1 h30. The residue obtained after solvent evaporation was purified by column chromatography on silica gel (CH$_2$Cl$_2$/MeOH 9:1, v/v). Product **8** was obtained as a white solid (304 mg, 60%).

Procedure C: **11** (170 mg, 1.1 mmol) and NEt$_3$ (700 µL, 5 mmol) were added to a solution of **14** (228 mg, 1 mmol) in THF (5 mL). The reaction was stirred until completion (usually 1h30) indicated by the disappearance of the yellow color due to the presence of **14** in solution. Concentration and purification by column chromatography on silica gel (ethyl acetate/MeOH 9:1, v/v) gave product **8** (76 mg, 34%) as a white solid.

Procedure D: **11** (51 mg, 0.33 mmol) and NEt$_3$ (100 µL, 0.7 mmol) were added to a solution of **15** (106 mg, 0.36 mmol) in dichloromethane (5 mL). The reaction was stirred for 20 h, concentrated under reduced pressure and purified by column chromatography on silica gel (ethyl acetate/MeOH 9:1, v/v) gave product **8** (10 mg, 13.5%) as a white solid.

Procedure E: A mixture of **11** (102 mg, 0.66 mmol) and NEt$_3$ (280 µL, 1.4 mmol) in dry MeOH (5 mL) was stirred for 15 min. Compound **16** (160 mg, 0.66 mmol) was then added and the mixture was stirred for 24 h, concentrated under reduced pressure and purified by column chromatography on silica gel (ethyl acetate/MeOH 9:1, v/v) gave product **8** (23 mg, 15%) as a white solid.

Melting point: 56.9–57.4 °C. TLC (CH$_2$Cl$_2$/ MeOH 9:1, v/v): R_f=0.3. ESI-MS (ES$^+$): m/z=226 [M+H]$^+$. ^{1}H NMR (400 MHz, CDCl$_3$): 1.54–1.63 (m, 2H), 1.67–1.82 (m, 3H), 1.96 (t, J=2.6 Hz, 1H), 2.11 (m, 1H), 2.23 (td, J=7.0 Hz, 2.6 Hz, 2H), 2.25–2.44 (m, 2H), 3.51–3.62 (m, 3H), 3.70 (m, 1H), 4.33 (m, 1H), 4.45 (m, 1H). ^{13}C NMR (100 MHz, CDCl$_3$): 18.7, 24.3, 28.4, 35.2, 37.5, 56.7, 60.3, 66.9, 69.2, 69.5, 84.6, 175.0.

Crystallographic data (Fig. 2): a=4.907(1) Å, b=7.353(1) Å, c=37.254(3) Å, V=1344.2(3) Å^3, orthorhombic, P2$_1$2$_1$2$_1$, Z=4, MoK$_\alpha$, μ=0,089 mm^{-1}, ρ=1.202 g cm^{-3}, F(0 0 0)=528, 5771 reflections (Rint=0.0195) from which 1769 unique and 1639 observed ($I > 2\sigma(I)$), R_1 (all reflections)=0.0484, R_1 (observed reflections)=0.0443, wR_2 (observed reflections)=0.1048, S=1.110, residual density (ΔF) 0.181 and $-$0.145 e Å^{-3}. Structure was solved and refined with the Shelxl package.

1-((2S,4R)-2-((dimethoxytrityloxy)methyl)-4-hydroxy-1-pyrrolidinyl)-1-(6-heptyn)one (9)

NEt$_3$ (2.77 mL, 20 mmol), DMAP (1.22 g, 10 mmol) and DMTrCl (4.6 g, 13 mmol) were addedto a solution of **8** (2.34 g, 10 mmol) in acetonitrile (20 mL). The mixture was stirred overnight at room temperature. The solvent was evaporated and the residue was purified by chromatography on aluminum oxide 90 active neutral (Toluene/acetone/NEt$_3$ 18:1:1 v/v/v). Product **9** was obtained as a brownish oil (830 mg, 15%). TLC (ethyl acetate 100%): R_f=0.35; ESI-MS (ES$^+$): m/z=303 [DMTr]$^+$ (100%), m/z=528 [M+1]$^+$ (10%), m/z=550 [M+Na]$^+$ (40%); m/z=566 [M+K]$^+$ (80%). ^{1}H NMR (400 MHz, (CD$_3$)$_2$CO): 1.61 (m, 2H), 1.77 (m, 2H), 1.93 (s, 1H), 2.13 (t, J=7.5 Hz, 2H), 2.23 (t, J=7.5 Hz, 2H), 2.29 (m, 2H), 3.14–3.26 (m, 2H), 3.43 (m, 1H), 3.49 (m, 1H), 3.72 (m, 1H), 3.79 (s, 6H), 4.62 (m, 1H), 6.82 (m, 4H), 7.25 (m, 7H), 7.37 (m, 2H). ^{13}C NMR (100 MHz, (CD$_3$)$_2$CO): 18.6, 24.4, 28.5, 34.8, 38.5, 55.5, 56.6, 63.8, 68.8, 69.3, 70.6, 84.4, 86.1, 113.4, 126.9, 128.0, 128.5, 129.4, 136.4, 145.3, 158.6, 171.3.

4-((3S,5R)-5-((dimethoxytrityloxy)methyl)-1-(6-heptynoyl)-3-pyrrolidinyloxy)-4-oxobutanoic acid (10)

A round-bottomed flask was charged with compound **9** (700 mg, 1.3 mmol), succinic anhydride (390 mg, 3.9 mmol), DMAP (158 mg, 1.3 mmol), NEt$_3$ (361 μL, 2.6 mmol) and 10 mL of dry acetonitrile. The mixture was stirred at room temperature overnight. Solvent was evaporated and residue was purified by preparative column on Kromasil® (MeOH/H$_2$O 7:3 v/v; 60 mL/min). Product **10** was obtained as a brownish foam (214 mg, 26%). Analytical HPLC: t_R=2.34 min (MeOH/H$_2$O 7:3, 1 mL/min); ESI-MS (ES$^+$): m/z=303 [DMTr]$^+$ (10%), m/z=628 [M+1]$^+$ (20%), m/z=650 [M+Na]$^+$ (15%), m/z=760 [M+NEt$_3$+MeOH]$^+$ (100%). ^{1}H NMR (400MHz, MeOD): 1.57 (m, 2H), 1.72 (m, 2H), 2.12 (s, 1H), 2.21 (m, 2H), 2.36 (m, 2H), 2.54 (m, 2H), 2.56 (m, 2H), 3.23 (m, 2H), 3.32 (m, 2H), 3.36 (m, 2H), 3.77 (m, 1H), 4.29 (m, 1H), 6.90 (m, 4H), 7.28 (m,7H), 8.11 (m, 2H). ^{13}C NMR (100 MHz, MeOD): 18.8, 25.2, 29.2, 30.5, 30.8, 31.2, 31.9, 35.3, 52.2, 55.8, 57.2, 57.8, 74.6, 108.2, 114.1, 127.8, 128.4, 129.2, 137.3, 146.4, 160.1, 174.3, 177.9, 178.4.

(2S,4R)-4-hydroxyprolinol hydrochloride (11)

Trans-4-hydroxy-L-proline **1** (5 g, 38 mmol) was suspended in acetonitrile (50 mL) and a mixture of hexamethyldisilizane (26 mL, 120 mmol) and trimethylsilylchloride (1.3 mL, 7.6 mmol) was added. After stirring for 9 h at reflux, the mixture was filtered and evaporated. The yellow oil obtained was dissolved in dioxane (200 mL) under an atmosphere of N$_2$. BH$_3 \cdot$SMe$_2$ (19 mL, 200 mmol) was added. The mixture was then stirred for 10 h at reflux and allowed to cool to room temperature. 1 M HCl (19 mL) was added dropwise and the mixture was heated to reflux for 1 h. The solvent was evaporated and co-evaporated with 1% conc. HCl in methanol (3 × 150 mL). Product **11** was obtained as a white solid (5.7 g, 96%). Melting point: 108.9–109.3 °C. ESI-MS (ES$^+$): m/z=118 [M+H]$^+$. ^{1}H NMR (400 MHz, CD$_3$OD): 2.05 (m, 1H), 2.23 (m, 1H), 3.41 (d, J=12.7 Hz, 1H), 3.53 (dd, J=3.8 Hz, 12.7 Hz, 1H), 3.80 (dd, J=6.9 Hz, 12.5 Hz, 1H), 4.01 (dd, J=3.5 Hz, 12.5 Hz, 1H), 4.12 (m, 1H), 4.75 (m, 1H). ^{13}C NMR (100 MHz, CD$_3$OD): 36.7, 54.4, 61.6, 61.7, 71.1.

1-(6-heptynoyl)pyrrolidine-2,5-dione (13)

TSTU (5.72 g, 19 mmol) was dissolved in acetonitrile (50 mL) and the solution was added to a solution of 6-heptynoic acid **12** (2 g, 16 mmol), NEt$_3$ (4.44 mL, 32 mmol) in CH$_2$Cl$_2$ (50 mL). The mixture was stirred for 30 min at room temperature, diluted with ethyl acetate (100 mL), washed with H$_2$O (2 × 100 mL), brine (75 mL), and dried over anhydrous MgSO$_4$. Product **13** was obtained as an yellowish foam. (3.76 g, 91%) Melting point: 62.9–63.5 °C. (ES$^+$): m/z=224 [M+H]$^+$. ^{1}H NMR: (400 MHz, CDCl$_3$): 1.55–1.66 (m, 2H), 1.88–1.79 (m, 2H), 1.94 (t, J=2.6 Hz, 1H), 2.21 (td, J=7.4 Hz, 2.6 Hz, 2H), 2.61 (t, J=7.4 Hz, 2H), 2.79 (s, 4H).

1-(2-thioxothiazolidine-3-yl)hept-6-yn-1-one (14)

Oxalyl chloride (1.5 g, 12 mmol) and 2 drops of DMF were added to a solution of 6-heptynoic acid (1 g, 8 mmol) in dry toluene (10 mL). The mixture was stirred until the end of gas production (3 h).The solvent was evaporated and co-evaporated with toluene (2 × 25 mL). The yellow oil obtained was diluted in anhydrous dichloromethane (20 mL) and triethylamine (2.4 g, 24 mmol). Thiazolidinethione (TTH) (1.43 g, 12 mmol) was added and the yellow mixture was stirred for 3 h. The organic phase was washed with brine (3 × 30 mL), dried with anhydrous MgSO$_4$ and evaporated. Yellow oil was obtained and purified by column chromatography on silica gel (CH$_2$Cl$_2$/ethyl acetate: 19/1). Product **14** was obtained as an yellow oil (693 mg, 38%). (ES$^+$): m/z=244 [M+H]$^+$. TLC (CH$_2$Cl$_2$/ethyl acetate: 19/1): R_f=0.9. ^{1}H NMR (250 MHz, CDCl$_3$): 1.51–1.59 (m, 2H), 1.67–1.70 (m, 2H), 1.86–1.89 (m, 2H), 1.91–1.93 (m, 2H), 2.20 (s, 1H), 3.26 (t, J=5 Hz, 2H), 4.54 (t, J=5 Hz, 2H).

Pentafluorophenyl hept-6-ynoate (15)

Oxalyl chloride (1.5 g, 12 mmol) and 2 drops of DMF was added to a solution of 6-heptynoic acid (1 g, 8 mmol) in dry toluene (10 mL). The mixture was stirred until the end of gas production (3 h).The solvent was evaporated and co-evaporated with toluene (2 × 25 mL). The yellow oil obtained was diluted in anhydrous dichloromethane (20 mL). Pentafluorophenol (1.4 g, 7.6 mmol) was added and allowed to cool to 0 °C. Triethylamine (1.6 g, 16 mmol) was added dropwise. The mixture was stirred at room temperature and, after 1 h30, diluted with dichloromethane (50 mL). The organic phase was washed with HCl 0.1M (2 × 40 mL) and brine (40 mL), dried with anhydrous Na$_2$SO$_4$ and evaporated. Brownish oil was obtained and purified by column chromatography on silica gel (Hexane/ethyl acetate: 4/1). Product **15** was obtained as a colorless oil (725 mg, 31%). (ES$^+$): m/z=293 [M+H]$^+$. TLC (Hexane/ethyl acetate: 4/1): R_f=0.73. ^{1}H NMR (250 MHz, CDCl$_3$): 1.54–1.74 (m, 2H), 1.81–1.93 (m, 2H), 1.95 (t, J=2.7 Hz, 1H), 2.25 (td, J=2.7 Hz, 6.9 Hz, 2H), 2.69 (t, J=6.9 Hz, 2H).

1-(1H-1,2,3-benzotriazole-1-yle)hept-6-yn-1-one (16)

1H-benzotriazole (1.9 g, 16 mmol) was added to a solution of SOCl$_2$ (476 mg, 4 mmol) in dry dichloromethane (20 mL) and the mixture was stirred 30 min. 6-heptynoic acid (504 mg, 4 mmol) was added and the stirring was continued overnight. The solution was filtered and the organic phase was washed with NaOH 1M (3 × 50 mL) and brine (30 mL), dried with anhydrous MgSO$_4$ and evaporated. The product was purified by column chromatography on silica gel (Hexane/ethyl acetate: 4/1). Product **16** was obtained as a white solid (320 mg, 33%). (ES$^+$): m/z=244 [M+H]$^+$. TLC (Hexane/ethyl acetate: 4/1): R_f=0.4. ^{1}H NMR (250 MHz, CDCl$_3$): 1.63–1.78 (m, 2H), 1.89–2.09 (m, 3H), 2.29 (td, J=7 Hz, 2.6 Hz, 2H), 3.45 (t, J=7 Hz, 2H), 7.49 (t, J=7.7 Hz, 1H), 7.63 (t, J=7.7 Hz, 1H), 8.10 (d, J=8.3 Hz, 1H), 8.27 (d, J=8.3 Hz, 1H).

(2R,3R)-threoninol (18)

L-threonine **17** (10 g, 84 mmol) was suspended in acetonitrile (150 mL) and a mixture of hexamethyldisilizane (54.2 mL, 260 mmol) and trimethylsilylchloride (2.16 mL, 17 mmol) was added. After stirring for 9 h at reflux, the mixture was filtered and evaporated. Yellow oil was obtained. The residue was dissolved in dioxane (150 mL) and under a N$_2$ atmosphere; BH$_3 \cdot$SMe$_2$ (41.4 mL, 436 mmol) was added. The mixture was then stirred for 10 h at reflux and allowed to cool to room temperature. 1M HCl (80 mL) was added dropwise and the mixture was heated to reflux for 1 h. The solvent was evaporated and co-evaporated with 1% conc. HCl in methanol (3 × 150 mL). Product **18** was obtained as a white oil (13.7 g, quant.). ESI-MS (ES$^+$): m/z=106 [M+H]$^+$ (85%); m/z=147 [M+H+CH$_3$CN]$^+$ (100%). ^{1}H NMR (400 MHz, D$_2$O): 1.19 (d, J=6.5 Hz, 3H), 2.69 (dt, J=6.8 Hz, 5.1 Hz, 1H), 3.49 (dd, J=11.3 Hz, 6.8 Hz, 1H), 3.65 (dd, J=11.3 Hz, 5.1 Hz, 1H), 3.73–3.82 (m, 1H). ^{13}C NMR (100 MHz, D$_2$O): 16.5, 54.8, 61.0, 65.8.

N-((2R,3R)-1,3-dihydroxy-2-butanyl)-6-heptynamide (19)

Compound **18** (4.42 g, 32 mmol) was dissolved in dry MeOH (120 mL) and NEt$_3$ (6.7 mL) and the solution was stirred for 15 min at room temperature. Compound **13** (3.5 g, 16 mmol) was added and the mixture was stirred for 1 h30. The solvent was evaporated and residue was purified by column chromatography on silica gel (CH$_2$Cl$_2$/ MeOH 9:1, v/v). Product **19** was obtained as a white oil (1.70 g, 50%). TLC (CH$_2$Cl$_2$/MeOH 9:1 v/v): R_f=0.1. ESI-MS (ES$^+$): m/z=214 [M+H]$^+$. ^{1}H NMR (400 MHz, CDCl$_3$): 1.14 (d, J=6.3 Hz, 3H), 1.52 (dt, J=15.3 Hz, 7.0 Hz, 2H), 1.72 (dt, J=15.3 Hz, 7.5 Hz, 2H), 1.94 (t, J=2.5 Hz, 1H), 2.18 (td, J=7.0 Hz, 2.5 Hz, 2H), 2.26 (t, J=7.5 Hz, 2H), 3.69 (d, J=4.7 Hz, 2H), 3.78 (s, 1H), 4.15–4.08 (m, 1H), 6.62 (d, J=8.4 Hz, 1H). ^{13}C NMR (100 MHz, CDCl$_3$): 18.2, 20.3, 25.0, 28.0, 36.1, 55.1, 63.9, 67.6, 68.9, 84.2, 174.7.

N-(((2R,3R)-1-dimethoxytrityloxy)-3-hydroxy-2-butanyl)-6-heptynamide (20)

TEA (1 mL, 12 mmol), DMTrCl (2.55 g, 7.5 mmol) and DMAP (22 mg, 0.18 mmol) were addedto a solution of **19** (1.28 g, 6 mmol) in dry CH$_3$CN (10 mL). The mixture was stirred 4 h at room temperature. The solvent was evaporated and the residue was purified by chromatography on silica gel (toluene/acetone/NEt$_3$ 88:7:5, v/v/v). Product **20** was obtained as a white oil (250 mg, 8%). TLC (toluene/acetone/NEt$_3$ 88:7:5, v/v/v): R_f=0.2. ESI-MS (ES$^+$): m/z=303 [DMTr]$^+$ (100%), m/z=538 [M+ Na]$^+$ (25%). ^{1}H NMR (400 MHz, CDCl$_3$): 1.17 (d, J=6.4 Hz, 3H), 1.59 (m, 2H), 1.81 (m, 2H), 1.99 (s, 1H), 2.24 (m, 4H), 3.31 - 3.4 (m, 2H), 3.77 (s, 6H), 4.01 (m, 1H), 4.13 (m, 1H), 6.30 (d, J=8.8 Hz, 1H), 6.87 (d, J=8.9 Hz, 4H), 7.15–7.46 (m, 9H). ^{13}C NMR (100 MHz, CDCl$_3$): 17.9, 19.8, 24.6, 27.6, 35.8, 53.4, 54.8, 64.6, 67.9, 68.6, 83.7, 86.4, 113.0, 125.0, 126.7, 127.7, 127.9, 135.4, 144.1, 158.1, 172.8.

4-((2R,3R)-4-(dimethoxytrityloxy)-3-(6-heptyn)amido-2-butanyloxy)-4-oxobutanoic acid (21)

A round-bottomed flask was charged with compound **20** (110 mg, 0.2 mmol), succinic anhydride (26 mg, 0.22 mmol), DMAP (1 mg, 0.01 mmol), and 10 mL of pyridine. The mixture was stirred at 55 °C for 4 h. The solvent was evaporated and the residue was purified by column chromatography on silica gel (toluene/acetone/NEt$_3$ 76:19:5 v/v/v). Product **21** was obtained as a white oil (80 mg, 61%).TLC (toluene/acetone/NEt$_3$ 76:19:5 v/v/v): R_f=0.4; ESI-MS (ES$^+$): m/z=303 [DMTr]$^+$ (40%), m/z=638 [M+Na]$^+$ (15%), m/z=717 [M+NEt$_3$]$^+$ (100%). ^{1}H NMR (400 MHz, CD$_3$OD): 1.07 (d, J=5 Hz, 3H), 1.52–1.57 (m, 2H), 1.70–1.79 (m, 2H), 2.17 (s, 1H), 2.15–2.20 (m, 2H), 2.26–2.32 (m, 2H), 3.07–3.12 (m, 1H), 3.30–3.32 (m, 5H), 3.77 (s, 6H), 3.97–3.99 (m, 2H), 6.85 (d, J=7.5 Hz, 4H), 7.18–7.44 (m, 9H). ^{13}C NMR (100 MHz, CDCl$_3$); 17.2, 18.8, 26.2, 29.2, 36.4, 40.2, 47.6, 54.0, 55.8, 63.4, 69.9, 70.7, 84.7, 87.4, 108.1, 114.1, 127.9, 128.8, 131.3, 137.1, 146.3, 160.1, 173.8, 176.0, 178.3.

4.3. Synthesis of siRNA

Sense-strand (22)

This compound was synthesized according to the literature. (Mercier et al., 2011). The synthesis was performed at Eurogentec (Liège, Belgium).

SiRNA modified (23)

The synthesis of antisense-strand was performed at Eurogentec (Liège, Belgium). The resin load was 33 μmol/g. These syntheses and the annealing between these two strands have been described by Mercier et al. (2011). The double-stranded siRNA **23** was obtained as lyophilized form.

4.4. Click chemistry

Synthesis of 1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{19}F]24

This compound was synthesized according to the literature. (Thonon et al., 2009).

Synthesis of siRNA [^{19}F]25

Azide [**^{19}F]24** (66 μL of a 40.8 mM stock solution in Me$_2$SO/t-BuOH: 3/1), CuSO$_4$.5H$_2$O/TBTA ligand complex 1/1 (21.6 μL of a 0.05 M stock solution in H$_2$O/Me$_2$SO/t-BuOH: 13.5/3.5/3, v/v/v), sodium ascorbate (10.8 μL of a 0.8 M stock solution in H$_2$O) were added to the double-stranded siRNA **23** (54 nmol in 18 μL of water). The mixture was stirred for 2 h at room temperature. The product obtained, [**^{19}F]25,** was purified by analytical HPLC, t_R=14.5 min, and finally lyophilized. Analytical HPLC: t_R=14.48 min. ESI-Q-ToF spectroscopy: 13692.001 (double-stranded), 7037.000 (sense), 6656.001 (antisense).

4.5. Radiochemistry

Synthesis of 1-(azidomethyl)-4-[^{18}F]fluorobenzene [^{18}F]24

This compound was synthesized according to the literature. (Thonon et al., 2009).

Synthesis of [^{18}F]siRNA [^{18}F]25

The double-stranded siRNA **23** (20 nmol) in H$_2$O (12 μL), sodium ascorbate (4 μL, 0.8 M) in H$_2$O, CuSO$_4$.5H$_2$O/TBTA ligand complex 1/1 (8 μL, 0.05 M) in Me$_2$SO/H$_2$O: 1/3 (v/v) was added to the solution of 1-(azidomethyl)-4-[^{18}F]fluorobenzene [**^{18}F]24** (200 mBq) in Me$_2$SO (0.5 mL). The mixture was stirred at room temperature for 15 min. After dilution with HPLC solvent (4 mL), the crude product was purified on a semi-preparative HPLC column (Jupiter Proteo 90 Å). The fraction containing the pure [^{18}F]siRNA [**^{18}F]25** (rt=20.2 min) was collected and diluted with water (50 mL), trapped on tC18 plus Sep Pak cartridge, eluted with ethanol (1 mL) and finally transferred onto NAP10$^®$ (*GE Healthcare*) Sephadex G-25 DNA grade column. The [^{18}F]siRNA [**^{18}F]25** was eluted with physiological solution to obtained 10 mL of injectable in pharmaceutical form.

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