UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Scuola di Dottorato in Scienze Mediche Sperimentali e Cliniche

Dottorato di Ricerca in TECNOLOGIE BIOMEDICHE (MED/50), XXVIII ciclo

F-18 RADIOLABELLING OF BIOLOGICALLY INTERESTING MOLECULES VIA "CLICK-CHEMISTRY"

Tutor: Dott. Sergio TODDE

Coordinatore: Prof.ssa Marina DEL PUPPO

Barbara LUGATO

Matr. n. 774791

Anno Accademico 2014-2015

CONTENTS

ABSTRACT

The doctorate project is focused on the development and standardization of a general, automated method for F-18 radiolabelling of biologically interesting molecules (e.g. peptides and oligomers), to carry out *in vivo* imaging assays by PET (Positron Emission Tomography).

The selected procedure deals with the introduction of a terminal alkynyl group into the biological substrate, and the synthesis of a functionalizable F-18 molecule containing an azide moiety. Once introduced the radioisotope, the following Huisgen [3+2] dipolar cycloaddition between the alkyne and the azide affords the desired radiolabelled biomolecule.

After problematic and unproductive attempts in employing commercially available or fully described azides, a completely new azido precursor useful for radiofluorination with F-18 was designed. The final radiolabelled azide is composed by a bifunctionalized aryl bringing, not directly linked to the ring, an azide group at one side, and a PEG chain terminated by a fluorine atom at the other. The aim was to obtain a precursor with suitable characteristics of versatility, easy handling, stability, and reactivity, necessary to make efficient conjugation to biomolecules, and to minimize non-specific binding during PET exam.

The synthetic work was carried-out in BIOMETRA Department laboratories of *Università degli Studi di Milano*, in collaboration with Prof. Patrizia Ferraboschi research group, while the radiochemistry activities were performed in laboratories of University of Milano-Bicocca, managed and operated by Tecnomed Foundation. Various synthetic pathways were tested in order to obtain an azido precursor useful for F-18 introduction, and for the subsequent cycloaddition reaction. In particular, a strategy of consecutive orthogonal protection-deprotection steps brought to the synthesis of three different precursor types (tosylate, mesylate, and iodinated), and the fluorinated reference molecule.

Starting from commercially available methyl 4-(bromomethyl)benzoate, the ester group was reduced to alcohol. The alcohol residue was protected as tetrahydropyranyl ether, while bromine was employed as leaving group to introduce a triethylene glycol chain. After protection of the terminal hydroxyl as acetate, benzylic hydroxyl was transformed to mesylate, and finally to azide. Removal of the acetate made the introduction of the mentioned leaving groups possible.

F-18 radiolabelling tests, carried-out on a dedicated automated synthesis module, demonstrated that the best precursor, in terms of radiolabelling yield and effectiveness of purification by separation to

the substrate, was the iodinated one. Purification of radioactive azide through cartridges procedure resulted in 51% radiochemical yield non-corrected for the decay (93% radiochemical purity).

It was then carried-out the conjugation of fluoroazide to a cost-effective biological model, propargylglycine. The "cold" click reaction was performed in a water/acetonitrile/DMSO solution, in presence of copper (II) sulfate and sodium ascorbate, bringing successfully to the corresponding 1,2,3-triazole. The radioactive analogue reaction showed the effectiveness of the conversion to the radioactive triazole (52% conversion, non-corrected for the decay) within 30 minutes.

In future developments, a resources scale-down concerning the radioactive procedure will be implemented. Moreover, the 1,2,3-triazole final purification method will be optimized, and this methodology will be applied to biologically relevant molecules (e.g. propargylglycine *S*-enantiomer or interesting "tool" peptides such as RGD or NGR). *In vivo* imaging will be performed on a dedicated small animal PET scanner, in collaboration with the pre-clinical research group directed by Prof. Rosamaria Moresco (University of Milano-Bicocca).

INTRODUCTION

The increasing application of Positron Emission Tomography (PET) in nuclear medicine for molecular imaging stimulated the demand for new and more advantageous method of radiolabelling. Between the wide variety of β+ emitter radioisotope useful for PET, emerged F-18, since its discovery in 1937. Actually, F-18 is the most widely used radionuclide in PET, because of its favourable characteristics in terms of decay and emission properties, high positron abundance (97%), relatively short half-life ($t_{½}$ = 109,8 min), lower positron energy (maximum 635 keV), and the shortest positron linear range in tissues giving the highest resolution (down to 1 mm in favourable conditions, i.e. micro-PET) during PET exam. For these reasons, F-18 labelled radiopharmaceuticals guarantee a lower radiation dose to patients, and a more accurate final image. Moreover, the half-life allows time-consuming multi-step radiosynthesis and the use of PET tracers with moderately slow pharmacokinetics. Finally, as F-18 can be produced in high yields, even with low-energy cyclotrons, it can be shipped and used in clinics that don't possess a cyclotron.

Radionuclide	**Half-life**	**Decay (% β+)**	**Maximal Positron Energy (MeV)**	**Theoretical Specific Activity (Ci/μmol)**
^{11}C	20.4 min	99	0.960	9215
^{13}N	10.0 min	100	1.19	18800
^{15}O	2.07 min	100	1.72	90960
^{18}F	109.8 min	97	0.635	1712
^{76}Br	16.1 h	57	3.98	193
^{124}I	4.18 d	24	2.13	31

Table 1

In general, the functionalization of a target molecule *via* the formation of a covalent bond between F-18 atom and the molecule of choice is preferred, because of the stability of the final radiolabelled compound, nevertheless other methods (as the use of bifunctional chelating complexes) are widely

used. Concerning covalent bond formation, in most cases radiofluorination means the bioisosterical exchange of a single hydrogen atom or a hydroxyl residue in the molecule of interest. Fluorine is absent in most biologically active compounds, and the replacement of residues with this atom causes only weak steric disturbances. In fact, fluorine Wan der Waals radius (1.47 Å) is only slightly larger than for protons (1.20 Å) and somewhat smaller than for oxygen (1.52 Å). Covalently bounded fluorine occupies smaller volume than methyl, amino, or hydroxyl groups, making incorporation favourable. Interestingly, the resulting C-F bond (453 kJ/mol) is stronger than the corresponding C-H bond (432 kJ/mol) or C-O bond (378 kJ/mol). Fluorine can also form hydrogen bonds, even if only as hydrogen bond acceptor in comparison with a hydroxyl group. However, a change in physiological and biological behaviour of the starting biological molecule is a consequence, as well as metabolic stability and lipophilicity.

There are two fundamental ways to covalently incorporate radioactive fluorine in the target molecule: the electrophilic and the nucleophilic one. In the electrophilic approach elemental molecular F-18 is used. However, the too high reactivity of $[^{18}F]F_2$ brings to poor selectivity, and this fact limited in practice electrophilic methodology application to reactions that require the direct introduction of F-18 in an aromatic ring. This methodology was often superseded by nucleophilic route, this bringing to high yield, high specific activity and selectivity. To date, nucleophilic methods are the most important concerning F-18 radiolabelling. Their predominance was also permitted by the advent of highly reactive F-18 fluoride species formed from F-18 aqueous anion. In fact, the most common and efficient way to produce F-18 is *via* the nuclear reaction $^{18}O(p,n)^{18}F$, which generate the activity in the form of fluoride anion in an aqueous media, where fluoride nucleophilic character is clearly too weak. Using tetraalkylammonium as counter-ions or complexating the alkali metal counter ion paired to F-18 anion by crown-ethers and cryptands (polyaminoethers, e.g. Kryptofix.222), the F-18 anion can be provided in a more reactive form.

The synthetic route by which F-18 anion is introduced in a substrate can be direct or indirect. Direct fluorination consists in a one-step procedure in which the simple radiolabelling of the precursor provides the desired radiolabelled product. Although this would be the preferred choice, it is unfortunately rarely applicable in practice. The indirect route usually consists in the radiolabelling of a precursor in which easily removable protecting groups are present, or in the direct radiolabelling of a small molecule providing a radiolabelled agent that can be coupled to the more labile molecule of interest. All the above indirect ways require a multi-step procedure that can be applied to F-18 because of its favourable half-life.

Nucleophilic radiofluorination can be divided in two categories: nucleophilic aliphatic substitutions and nucleophilic aromatic substitutions. The nucleophilic aliphatic substitution suits more often an

S_N2-mechanism, in which fluoride activated anion can replace a leaving group such as a halo, tosilate, mesylate, or a triflate group. As attended, the reactivity of fluorine substitution decreases from primary to tertiary carbon position. In this context, the most widely used PET radiopharmaceutical, [^{18}F]FDG (fluorodeoxyglucose, used to assess the glucose metabolism of a tissue), is currently produced by [^{18}F]fluoride substitution of a triflate group, followed by hydrolysis of all acetyl protecting groups. Concerning nucleophilic aromatic substitution, an S_NAr-mechanism is involved, in which [^{18}F]fluoride replace a leaving group directly on the aromatic ring, the latter being activated by an electron withdrawing group in *ortho*- or *para*- position to the leaving group. A general limit in S_NAr is the subsequent necessary removal or derivatization of the activating group, and the frequently poor radiofluorination selectivity. A recent development of this strategy was the employing of an asymmetric or symmetric diaryliodonium salt as precursor, with attack of the more electron deficient ring by fluorine to give the corresponding [^{18}F]F-labelled arene and the iodoarene. In this case, an *ortho* steric phenomenon between the substituents increases radiofluorination yields and the reaction proceeds in some cases even in slightly aqueous conditions.

In this context, macromolecules such as oligonucleotides, peptides, aptamers, oligonucleotides, peptide nucleic acids, proteins, antibodies, and others are receiving increasing attention as PET radiopharmaceuticals for both diagnostic and therapeutic applications. As direct radiofluorination is almost impossible, a variety of indirect methods were developed, the most common the radiofluorination of a small molecule (to achieved the so called “prosthetic group”), which can then be conjugated to the macromolecule under mild conditions, in respect of its labile functionalities. Also derivatization of small molecules often suited this approach. Figure 1 shows some representative example of ^{18}F- prosthetic reagents.

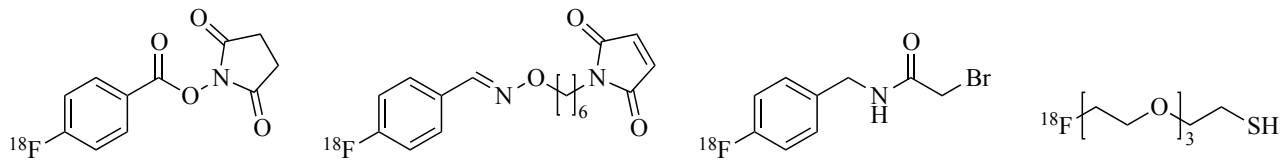


Figure 1

Alternative recently proposed prosthetic groups useful for radiofluorination of macromolecules were silicon and boroaryl-based [^{18}F]fluorine acceptors, modified or non-modified [^{18}F]FDG, Al[^{18}F] complexes, [1] and modified [^{18}F]N-heterocyclic carbene boron trifluoride adducts. [2]

Methods useful to introduce [^{18}F]fluorine in a macromolecule through a prosthetic group, can be carried-out by a conventional pool of reactions (acylation, alkylation, amidation, thiol-coupling), as well as less common approach (glycosylation, imidation, oxime and hydrazone formation, thiourea formation, photochemical conjugation) employing group naturally occurring in macromolecules (amino, carboxyl, thiol), or introduced by chemical modification. The first ^{18}F-labelled peptide for example was obtained from the conjugation of an ^{18}F-labelled ester with octreotide. The prosthetic group carrying the F-18 atom is usually attached at a well-defined position of the macromolecule, where it cannot perturb its biological function. However, a poor chemoselectivity during conjugation with the macromolecule usually occurs, and consequently it appears the need to protect these potential reacting groups, especially in peptides. The labile character of the formed linkage, and an increasing in the final lipophilicity of the molecule can represent a further limitation regarding this approach. For these reasons, alternative types of coupling reactions had to be investigated. [3] [4] [5] [6]

In this context, it was recently demonstrated the efficacy of "click" reactions. Among these, the Cu(I) catalyzed version of the Huisgen [3+2] dipolar cycloaddition between terminal alkynes and azides, the so called CuAAC (Copper-catalyzed Azide-Alkyne Cycloaddition), gave better results. This reaction showed an interesting list of positive characteristics that made it widely applied in the last years in various fields ranging from material science [7] [8] [9] to chemical biology, [10] [11] [12] [13] drug development, [14] and of course radiopharmaceutical chemistry. [15] [16]

First of all, it shows reduced reaction times, mild reaction conditions in aqueous media, excellent yields, and easy purification (poor by-products). Another advantage is the fact that alkynes and azides are almost inert towards classic functional groups, so there's no need of protection strategy during click reaction. Furthermore, the resulting product (the 1,2,3-triazole moiety) is particularly suitable to be employed in biologic media, because of its biological stability, non-toxicity, and polarity and size similar to amide or peptide bonds. [4]

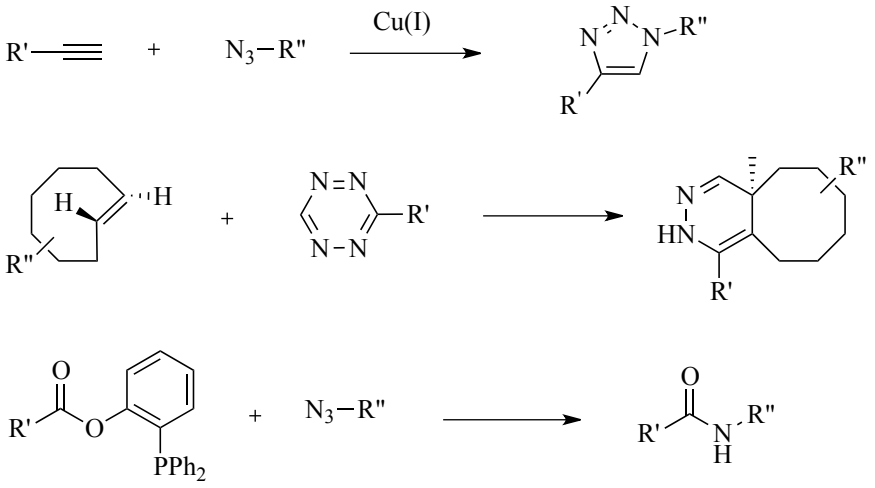
Concerning the triazole product, the click reaction shows high specificity by opportunely varying reaction conditions. The direct use of Cu(I) salts, for example CuI, CuBr, $CuOTf{\cdot}C_6H_6$, and $[Cu(NCCH_3)_4][PF_6]$, in the absence of a reducing agent usually require acetonitrile as co-solvent and one equivalent of a nitrogen base, such as 2,6-lutidine, triethylamine, diisopropylethylamine, or pyridine), and it often brings to the formation of various by-products, such as diacetylenes, bis-triazoles, and 5-hydroxytriazoles. The employment of 2,6-lutidine and the exclusion of oxygen minimize this complication. Furthermore, the produced triazole is present as a mixture of 1,4- and 1,5-disubstituted 1,2,3-triazole. A more reliable and simple method is the *in situ* reduction of Cu(II)

salts by a reductant (with or without co-solvents and amine or buffer additives), typically Cu(II) sulfate pentahydrate and ascorbic acid or sodium ascorbate as reductant. Cu(II) salts are less costly and often more pure than Cu(I) salts, and the only 1,4-disubstituted 1,2,3-triazole is often observed as unique regioisomer. This reaction does not seem to require any special precautions, and it proceeds to completion in 6-36 hours concerning classical chemical synthesis, in a variety of solvents including *tert*-butyl alcohol or ethanol, and, up to others, in aqueous media with or without a co-solvent (ethanol, acetonitrile, dimethylsulfoxide, *N*,*N*-dimethylformamide), in pH ranging from 4 to 12. [17]

This robust catalytic process is the element of choice around which this work of thesis was developed.

Sometimes the use of copper represents a problem because of the potential risk of injection of cytoxic Cu(I) ions, that cause oxidative degradation of DNA [18] [19] and proteins, [20] *via* hydroxyl radicals formation *in vivo*. Catalyst-free alternatives were developed, such as the strained version of the Huisgen [3+2] dipolar cycloaddition, the inverse Diels-Alder tetrazine-click reaction, and the Staudinger Ligation. The driving force in the strained Huisgen [3+2] dipolar cycloaddition is given by the energy relief in a strained cyclooctyne when the azide adds to the triple bond, but other 1,3 dipoles can be useful too, as the recently proposed nitrone function, which adds to alkenes without a catalyst. The inverse electron demand Diels-Alder cycloaddition employs a *trans*-cyclooctene and a tetrazine derivative to give a cyclooctapyridazine. This reaction is practically immediate and can be carried out in aqueous media; a similar reaction type is the photoactivated addition of a dipolarophilic alkene to a substituted tetrazole. The Staudinger ligation employs an organic azide that reacts with an appropriate phosphine activated compound, resulting in an amide bond. The phosphine part is eliminated from the aza-ylide intermediate as a phosphine oxide, after re-arrangement, leaving no trace in the product. These reactions present no-need for a catalytic system, and the produced linked moieties are metabolically stable under physiological conditions.

In Scheme 1 are represented the different click reaction types described above, used in fluorine radiochemistry. [4] [5]



Scheme 1

Other investigated possibilities are the radical addition between thiols and alkenes, the Michael addition of thiols with maleimide, and the nucleophilic substitution of the *p*-fluoro substituent of pentafluoro-phenyl groups.

In order to accelerate the azide-alkyne 1,3-dipolar cycloaddition, a wide variety of new ligands capable to stabilize Cu(I) were investigated, especially *N*-donor ligands, [21] as well as the use of other transition metals (Ru, [22] Ir, [23] Ni, Pd, Pt, [24] Ag, [25] Au, [26] Fe, [27] Zn [28]). In particular, zinc and ruthenium complexes were found to catalyze the formation of 1,5-disubstituted triazoles from azides and terminal alkynes.

Even if copper-free click reactions are somewhat promising and completely avoid copper cytotoxicity problem, CuAAC permits a larger choice in the design of useful precursors, and remains a simpler procedure, that is a fundamental issue concerning radiochemistry using automated synthesis systems. Moreover, methods involving ligands for copper or metal complexes often employ molecules and procedures of high complexity, and they are often more pH and solvent sensible. [29]

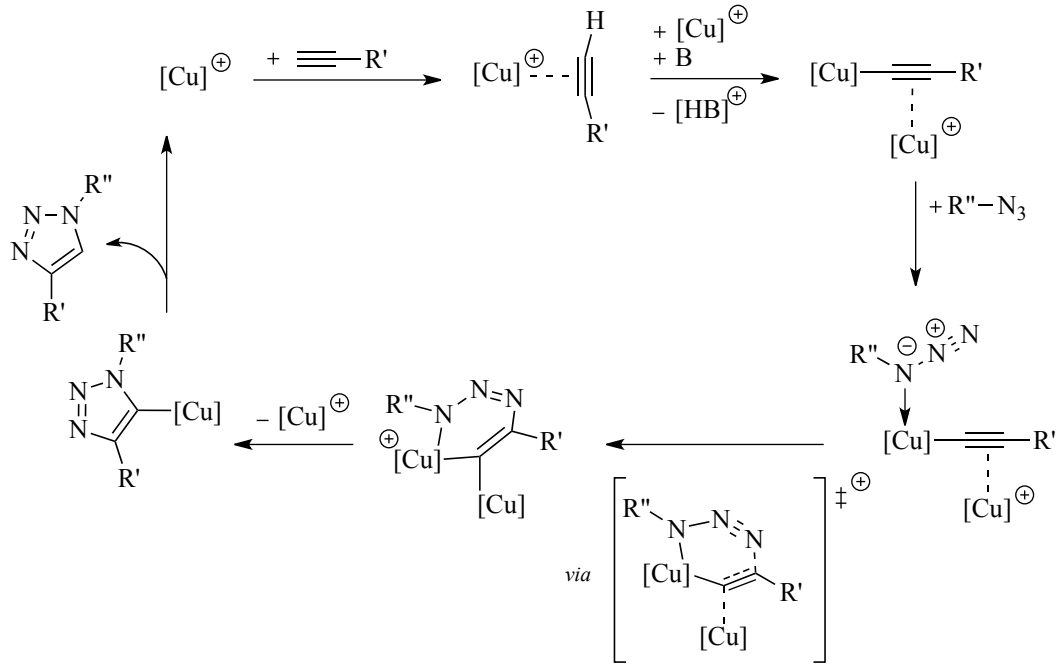
Focusing on CuAAC, among the three most common oxidation states of copper (0, +1, and +2), +1 is the least thermodynamically stable. Cuprous ion can be oxidized to catalytically inactive Cu(II) species or can disproportionate to a mixture of Cu(II) and Cu(0). When Cu(I) catalyst is used directly, whether by itself or in conjunction with amine ligands, exclusion of oxygen may be required. A revolutionary method that allowed by-passing of oxygen-free conditions and that was definitely accepted as the procedure of choice for preparative synthesis of 1,2,3-triazoles was introduced by Fokin and co-workers and consists in the use of sodium ascorbate as mild reductant, in conjunction with Cu(II) salts, such as the readily available and stable copper(II) sulfate

pentahydrate or copper(II) acetate. In this case, the Cu(I) ion is formed *in situ* and results to be very reactive, especially in water-alcoholic media, that reduces aggregation of the intermediate reactive state copper acetylide. This catalytic procedure is demonstrated to be highly regioselective, giving only the 1,4-disubstituted 1,2,3-triazoles, as mentioned, and accelerates the cycloaddition by a factor up to 10^7 in comparison to Huisgen uncatalyzed procedure. Moreover, the formation of byproducts resulting from copper-mediated oxidative side reactions is suppressed as any dissolved dioxygen is rapidly reduced. This procedure often provides triazole products in nearly quantitative yield and greater than 90% purity. (30)

Generally, only in aprotic organic media amine additives or bases are essential to deprotonate the alkyne substrate, whereas the formation of corresponding Cu(I) acetylides in aqueous media is so easy that it even occurs in strongly acidic solutions. (31)

It is clear that under ligand-free conditions, solvent molecules such as acetonitrile or nitrogen bases can coordinate to Cu(I) ions, thus ameliorating catalytic performance by preventing oxidation and disproportionation reaction, (32) as well as the formation of unreactive polynuclear Cu(I) aggregates. (33) (34)

Various diverging mechanistic theories around CuAAC were proposed and recently reviewed. (35) Among these, the most reliable mechanism concerning CuAAC employing *in situ* reduction of Cu(II) salts by ascorbate, without other additives, in a water environment is based on DFT calculations by Fokin (36) and Berg (37) groups. This is represented in Scheme 2 and discussed below.



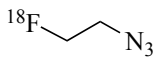
Scheme 2

Fokin and co-workers demonstrated that CuAAC reaction can only take place when at least two Cu(I) centres cooperate in the rate-determining C-N bond forming step. In the first step a Cu(I) ion π-coordinates the alkyne substrate, that can then be easily deprotonated, while another Cu(I) centre attracts the acetylide complex. It is in the following step, when both the azide and the σ-acetylide ligand are bounded at the same Cu(I) centre, that the formation of the C-N bond can occur. However, it remains an unresolved question whether the formation of triazoles after several hours can be attributed to a very slow process involving mononuclear species or to catalysis by traces of a reactive Cu(I) species, according to Fokin's proposed mechanism.

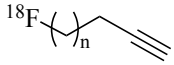
Regarding kinetic characteristics, the CuAAC reaction was empirically found to be second order in the concentration of Cu(I) ions under catalytic conditions. At high metal concentration, formation of aggregates occurs and the reaction was found to be between first and second order in the concentration of the alkyne. [38] Interestingly, the concentration of the azido substrate doesn't seem to influence the reaction rate. Other recent assays empirically confirmed that CuAAC rate depends on alkyne concentration at high Cu(I) concentration, also in radiofluorination context. [39]

Concerning CuAAC involving ligands, the kinetic was observed to change in dependence of the nature and the concentration of the ligand, on the presence of chloride ions, the type of buffer, and other parameters, showing the higher complexity and variability of this method compared to ligand-free CuAAC.

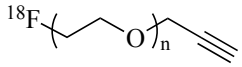
As a consequence of CuAAC improvement, a wide variety of new precursors were developed, also in radiofluorination field. Below (Figure 2) are reported some example of new ^{18}F-prosthetic groups that were employed in the last years in biomolecules radiolabelling. [40]



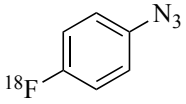
[^{18}F]fluoroethylazide
([^{18}F]FEA)



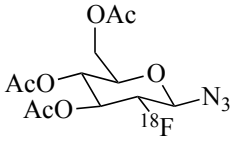
[^{18}F]fluoroalkyne



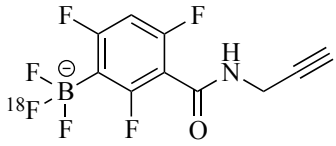
[^{18}F]fluoro-PEG-alkyne



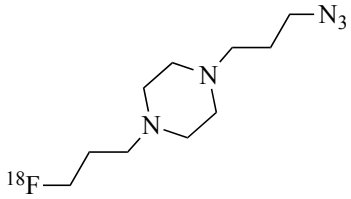
1-(azidomethyl)-4-[^{18}F]fluorobenzene



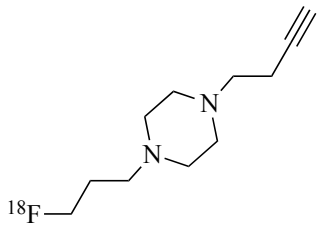
3,4,5-tri-*O*-acetyl-2-deoxy-
2-[^{18}F]fluoroglucopyranosyl azide



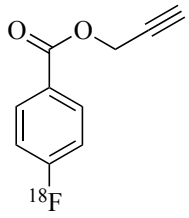
[^{18}F]ArBF$_3^-$



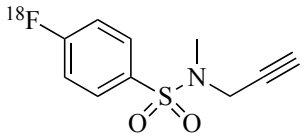
1-(3-azidopropyl)-4-(3-
[^{18}F]fluoropropyl)piperazine
([^{18}F]AFP)



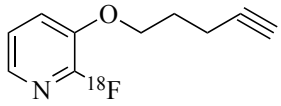
1-(but-3-ynyl)-4-(3-[^{18}F]fluoro-
propyl)piperazine
([^{18}F]BFP)



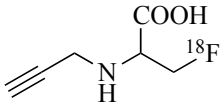
O-propargyl-4-[^{18}F]fluorobenzoate
([^{18}F]PFB)



4-[^{18}F]fluoro-*N*-methyl-*N*-(prop-2-ynyl)-
benzenesulfonamide
([^{18}F]F-SA)



[^{18}F]FPy5yne



N-propargyl-2-amino-3-
[^{18}F]fluoropropionic acid
([^{18}F]serine)

Figure 2

Among these, [^{18}F]FEA is surely one of the most investigated ^{18}F-prosthetic groups. It appears in about twenty different manuscripts concerning the radiolabelling of a broad variety of biomolecules and compounds. The first preparation of [^{18}F]FEA was carried out in 2007 by Glaser and Årstad from the corresponding tosylate, with a radiochemical yield of 55%. They reacted [^{18}F]FEA with different terminal alkynes obtaining radiochemical yields varying from 61 to 98%. [(16)] To reduce volatility and increase polarity, Sirion et Al. described the synthesis of the first [^{18}F]fluoro-PEG-derivative. [(41)] Other groups continued the work of Sirion et Al. by radiolabelling cRGD derivatives [(42)] and other peptides, [(43)] nanoparticles [(44)] [(45)] or folates. [(46)] The PEG chain makes not only easy to handle compounds, but it also improve the *in vivo* behaviour of labelled molecules by elongating

circulation time and reducing renal clearance. The same effect was reached with ^{18}F-gluco-derivatives, developed by Maschauer and Prante by Fmoc-*L*-propargylglycine labelling with 60% radiochemical yield. [47] They published the first *in vivo* evaluation of an ^{18}F-labelled RGD peptide using [^{18}F]FDG-β-Az in U87MG-tumor bearing mice showing an increased blood clearance and stability. [48] One of the latest interesting developments reported by Schieferstein and Ross involves the synthesis of a ^{18}F-labeled alanine as new prosthetic group capable to improve the pharmacokinetic profile of ^{18}F-click labelled biomolecules. [49] On the other hand, prosthetic group such as [^{18}F]AFP were developed to avoid the possible formation of strong complexes between the target peptide and the copper catalyst. [50] In fact, in order to allow *in vivo* application the removal of copper catalyst is fundamental.

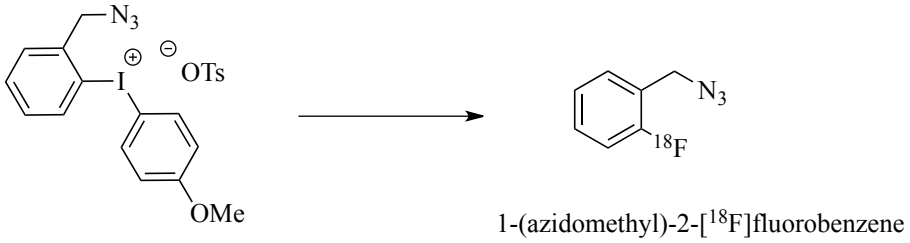
AIM OF THE WORK

The goal of this work is the development and optimization of a general synthetic procedure for F-18 radiolabelling of biologically interesting molecules. The radiolabelling procedure should be completely automated, in order to ensure radiation protection measures to operators, and to guarantee a robustness and repeatability of the synthetic procedure, thus saving time, resources, and allowing radioactive chemicals production suitable both for internal purposes and for pre-clinic evaluation, the latters currently having to be performed in a Centre located at a 20 Km distance. The radiolabelling procedure should be general enough to be transferred to a wide variety of biologically interesting macromolecules, especially peptides, oligomers, and aptamers, whose activity and metabolism are requested to be investigated, in view of their potential imaging applications.

The method of choice is the conjugation of an ^{18}F-functionalized prosthetic group to the opportunely modified biomolecule *via* ligand-free CuAAC. This kind of "click" cycloaddition seems to be particularly indicated for our purposes, because of its simplicity, high yields even in short reaction times, and mild conditions that respect biomolecule functional groups, as far discussed in the introduction session of this thesis.
In particular, standing on the finding that ligand-free CuAAC, in presence of an excess of metal catalyst, is kinetically rate-dependant on the amount of the alkyne substrate, we decided to ^{18}F-radiolabel an azido compound, and consequently employing a yet alkynyl-modified biomolecule. This decision was also influenced by the fact that azido-modified biomolecules are more expensive, more sensitive, and more difficult to purchase and transport than alkynyl analogues.

At the beginning we focused on commercially available azido precursors useful for ^{18}F-radiolabelling and CuAAC reaction. As our radiochemistry labs are not equipped to perform classic organic chemistry, also the cold reference compounds had to be purchased, in order to monitor radiolabelling procedure efficacy.
The first molecule we focused on was [^{18}F]FEA, because of its great description in literature and commercial availability. Only a few suppliers sell both the precursor and the fluorinated reference compound, and this is definitely comprehensible as these molecules are sensible and difficult to handle, because of their volatility and potential explosion danger. The tosylate derivative and the

cold reference molecule were purchased from a German supplier (Select Lab Chemicals GmbH, Bönen, Germany), but the first RP-HPLC analysis revealed that these products were already degraded from their coming. Subsequent radiochemical assays and ESI-MS analysis confirmed this finding, and it was clear that, because of their nature, these molecules have to be freshly synthesized and immediately used in your own labs. This route was thus abandoned, and we looked for a more stable and easier to handle molecule: what we focused on was 1-(azidomethyl)-2-[^{18}F]fluorobenzene and its precursor diaryliodonium tosylate salt (Scheme 3), supplied by an English company (Nimasol Limited, Newcastle upon Tyne, UK).



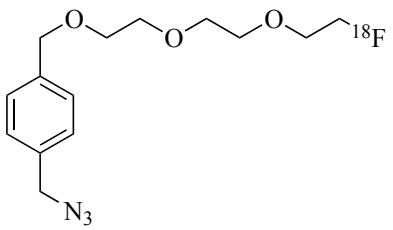
Scheme 3

However, in the end the supplier refused to ship these compounds until physical hazard tests should be performed, accordingly with EU regulation. These molecules were indeed suspected of thermal instability and, because of their chemical structure, to be unstable and subject to spontaneous decomposition with potentially explosive effects under certain circumstances. Other suppliers of similar products appear to be offering them for sale and apparently shipping them without any reliable knowledge of their physical hazard characteristics, but this company finally wouldn't take that risk. As a time schedule for test and shipment was not clearly defined, we decided to synthetize these compounds by ourselves in a suitably equipped organic chemistry lab, within a collaboration meanwhile established with the group lead by Prof. Ferraboschi. However, a careful analysis of the reaction conditions to be used made us to discard this synthetic route, because of the lack of available instrumentation for hazardous synthesis, also recommended by the authors. [(51) (52)]

We concluded that the lack of fluorinated reference and precursors concerns not only their commercial availability. In fact, those ones mentioned in literature present some negative characteristics, the dangerous synthetic procedure (i.e. purification by distillation of potentially explosive molecules or dangerous mixing of reagents) and the inherent instability of the intended compounds being the main ones. In general, organic azides are considered as explosives whenever the azido content is remarkably high. Of course, there is no sharp threshold at which the explosive hazard starts. However, as a rule of thumb, violent decomposition reactions are expected for azido

compounds having a (C + O)/N ratio of <3. [53] In this case the molecule can explode both for friction or heat, especially if it is isolated and dried, making necessary a proper handling of the substance. [54] Another negative recurrent characteristic of azido compounds employed in radiochemistry is the high degree of lipophilicity (occasionally moderated by adding a PEG chain), that can compromise CuAAC yield or eventually require more organic solvents to homogenize reagents in this step, and decrease *in serum* availability. Moreover, a low stability of intramolecular bonds can determine degradation of the radiolabelled molecule and *in vivo* formation of radiolabelled by-products, thus affecting imaging properties. UV detectability is sometimes poor, making even more difficult the development of radiolabeling and purification methods. Improved substrates, reference standard, and final products are very attractive in radiochemistry, but this kind of optimization is also difficult to achieve, because of the extended chemical work behind it. Therefore, groups tend to focus onto biological experiments and usually make a hurried work concerning chemical substrates. This is immediately clear by observing the wide variety of molecules described in literature in which the azido group or the polar chain are directly linked to an aromatic ring, in which the ^{18}F radioisotope is directly linked to the polar chain or the aromatic ring (a loss of stability of these functional groups is the more common consequence, and the azide is less available during CuAAC, that is therefore slowed-down), in which is absent an useful UV absorption character, or again that are too simple to satisfy more requirements.

Standing on these premises, we finally decided to point our attention to the design and synthesis of a completely novel precursor and related fluorinated reference standard, which would satisfy the above outlined requisites of versatility, stability, polarity, reactivity, strong UV absorption character, easy handling (Figure 3). As a consequence, the amount of alkynyl-modified biomolecule is expected to be lower than requested in current methods, and the radiolabelled biomolecule may result in improved sensitivity and specificity, because of the attended *in serum* stability and availability.



[^{18}F]1

Figure 3

The effort described in this thesis definitely consisted in the synthesis of a suitable precursor to obtain compound **[^{18}F]1**, the synthesis of the fluorinated reference standard, the development of an optimized ^{18}F-radiolabelling procedure, the CuAAC of the radiolabelled prosthetic group with a model, propargylGly, to test its efficacy and to provide a starting point to transfer the conjugation method to biologically interesting molecules. HPLC methods were developed to monitor radiochemical synthesis, and purifying procedures were optimized. Radiochemical procedures were fully automated to improve repeatability and robustness of radiolabelling methods.

Future developments will be the *scale-down* concerning alkyne amount in the CuAAC, the transfer of the method to biologically interesting molecules, and *in vivo* preclinical evaluations of the obtained radiolabelled compounds.

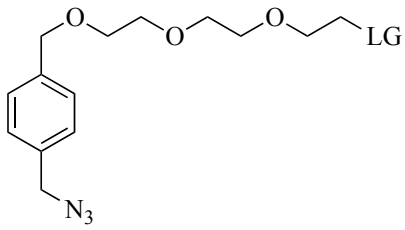
RESULTS AND DISCUSSION

1. CHEMICAL SESSION

Preparation of unlabelled compounds was carried out in the laboratories of Medical Biotechnology and Translational Medicine Department of *Università degli Studi di Milano*. Purification of the final fluorinated product, as well as RP-HPLC analysis, were performed in the laboratories of the University of Milano-Bicocca (Tecnomed Foundation).

1.1 CHEMICAL SYNTHESIS

In order to obtain the aforementioned radiolabelled product **[^{18}F]1**, we started by designing some potentially useful precursors (Figure 4), and projecting a strategy to achieved them. The difficult was to bifunctionalize in a different way the two benzylic positions of an aromatic substrate with different reactive functional groups. What got the procedure more complicated was the introduction of a triethylene glycol chain.



LG = -OTs (**2**), -OMs (**3**), -OTf (**4**), -I (**5**)

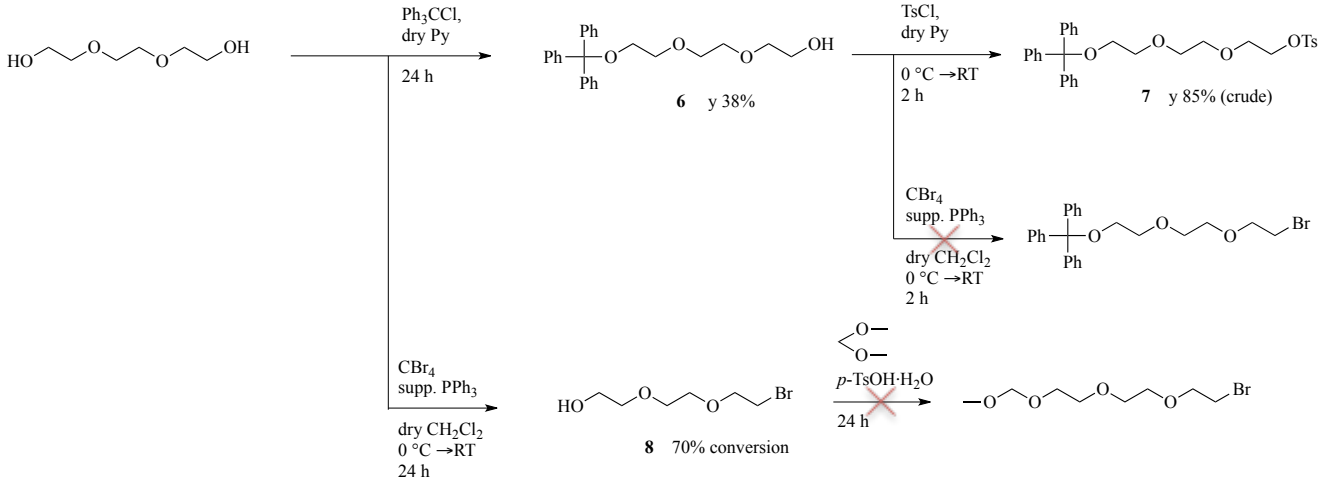
Figure 4

The synthetic pathway focused on an orthogonal protection-deprotection strategy of functional groups that is fully discussed below.

The key-concept was the selective orthogonal functionalization of the two benzylic positions on a starting arene moiety and of the two extremities of a PEG chain, such in a way that we should

selectively attach the PEG chain to the substrate, without affecting the remaining protecting groups, that should be resistant under coupling conditions. The removal of these protecting groups should allow the subsequent functionalization of the benzylic residue with an azido group, and then of the PEG terminal position with the desired leaving group to give products **2**, **3**, **4**, **5**. Of course, in order to obtain a successful synthetic procedure, the chemical nature of the different protecting groups had to be suitable for respect of functional groups and for reaction conditions under which they had to stay. Underlying this idea, once the azido group was formed, the subsequent reactions had to avoid acid conditions, because of the azide labile character in acidic media. [55] For this reason, the protecting group on the PEG chain had to be removable in conditions alternative to acidic ones and, standing on this assumption, the protecting group that should allow introduction of the azide moiety had to be orthogonal to this protecting group not removable in acidic conditions. It's clear that a well-defined nature of groups had to be assessed when undertaking whatever synthetic pathway, and that each step has a biunivocal relation with any other ones.

The first step in the development of such a synthetic pathway dealt with the investigation of protection possibilities on PEG chain. The assays are represented in Scheme 4.



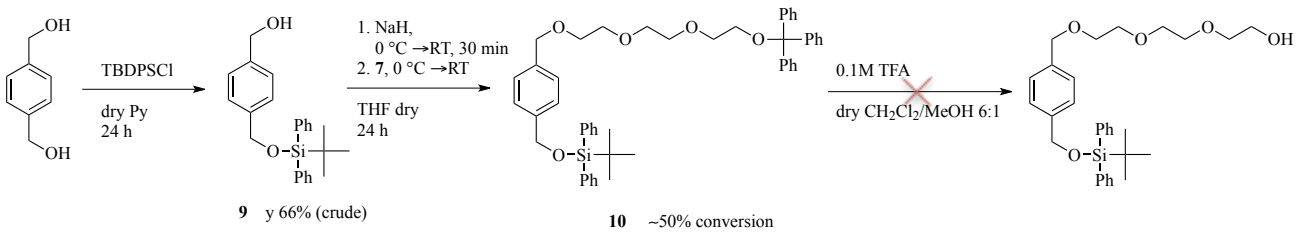
Scheme 4

Trityl group and methyl acetal were chosen as suitable protecting groups to be removed after the conjugation of PEG chain with the benzylic substrate, but before the functionalization of the other benzylic position with the azido group, as both are removable in acidic conditions and stable in basic ones. As bromination on compound **6** presented some problems, [56] only product **7** could be used in the first designed procedure that involved an aromatic ring bearing two hydroxymethyl moiety.

The alternative was to use compound **6** employing an aromatic substrate carrying in the benzylic positions a leaving group for conjugation with this one and a protecting group at *para* position. Compound **8** was set aside, because the impossibility to purify by column chromatography such a molecule carrying a bromine group, and the poor selectivity of possible reactions with the proposed substrates.

The commercial substrates we selected, somehow outlined above, are cost-effective methyl 1,4-benzenedimethanol (the substrate with two equal functionalities) and 4-bromomethyl benzoate (the substrate with two different functional groups).

Concerning methyl 1,4-benzenedimethanol, the synthetic route is shown in Scheme 5. Of course it was developed in dependence to what we observed in the synthetic way described in Scheme 4.



Scheme 5

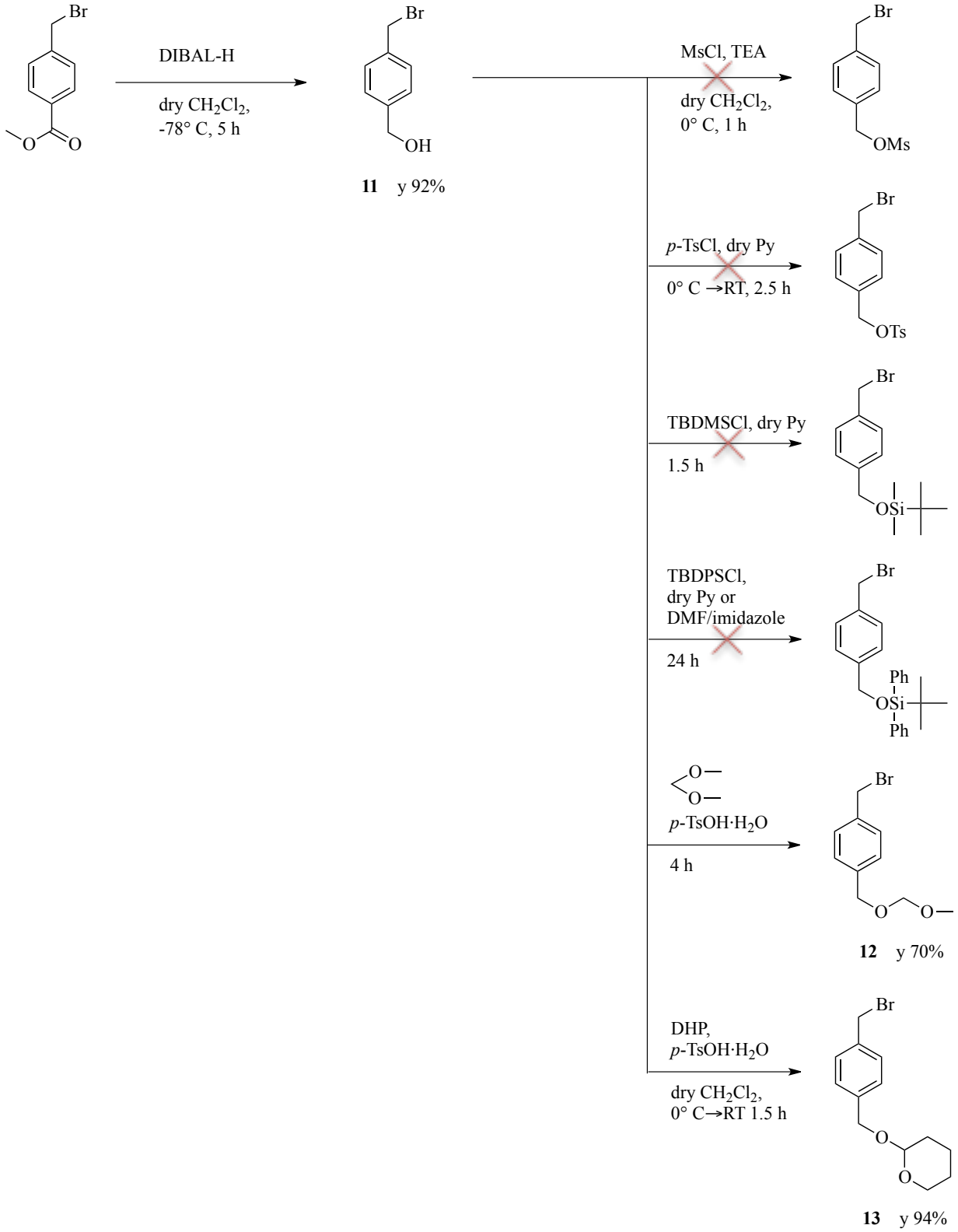
Trityl and silyl groups are both removable in acidic conditions, and stable in basic ones. Product **9** seemed to be easy to obtain, with a little residual amount of silylating agent, but the conjugation reaction with compound **7**, *via* ether formation, [57] brought to a very complex mixture that couldn't be purified. The consequent deprotection of oxydryl group from trityl was carried out in a mild acidic media that should not attack silyl group, theoretically removable in stronger acidic conditions. However, in practice, the employed conditions caused incomplete removal of trityl group and partial removal of silyl group, making infeasible this way.

Our efforts were therefore moved to the employment of 4-bromomethyl benzoate as starting substrate.

Whereas two different functionalities are just inside this molecule type, we could focus onto functionalization of the precursor maintaining unvaried or mono-functionalized the PEG chain. This concept will be better understood from following.

As first thing, the ester function in 4-bromomethyl benzoate was reduced to alcohol, according to an established procedure described in literature, [58] and the obtained hydroxyl group was variously

functionalized. In Scheme 6 the reduction of the substrate and functionalization assays are described.



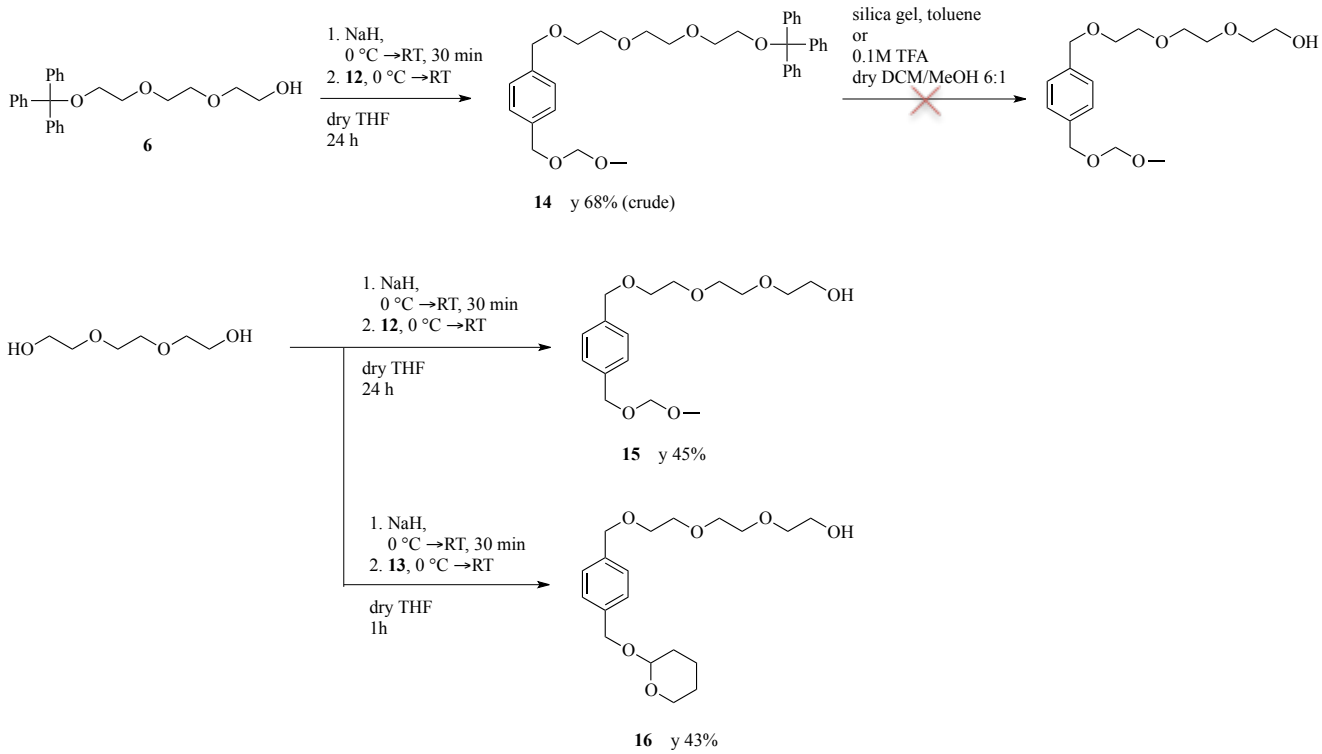
Scheme 6

Functionalization with leaving groups such as mesylate or tosylate, that should be replaced by a nucleophile in stronger conditions than bromine, bringing to following selective functionalization, was not feasible. During the introduction of tosylate, the salt formation between the product and pyridine was observed, bringing to a very poor yield and unusable product. Concerning mesylate synthesis, attack of bromine atom was observed meanwhile the formation of the product that soon degraded, bringing to complex mixtures. The protection of hydroxyl moiety, a longer but more guaranteed route, was then carried out. *Tert*-butyldimethylsilylation seemed to proceed well, but the product revealed to be too much volatile, compromising the yield. The analogue less volatile product, made from *tert*-butyldiphenylsilylation resulted again in a poor yield mixture of product and reagents in pyridine, and a more complex mixture concerning DMF/imidazole media, [59] probably due to reactivity of bromine moiety.

What we definitely founded as good reactions was the functionalization with formaldehyde dimethyl acetal, [60] the desired protected product **12** in a 70% yield after column chromatography purification, or the protection with 3,4-dihydro-2H-pyran, [61] 94% yield of compound **13**, after column chromatography purification.

Both protections of substrate as acetal and tetrahydropyranyl (THP) ether were thought to well sustain Williamson etherification between the substrate and the protected or non-protected PEG chain, and to be resistant during the subsequent removal of the eventual protecting group on the terminal PEG hydroxy group. In the latter case, the protected PEG chain was compound **6**, so the protecting group was the *tert*-butyldiphenylsilyl moiety. Acetal, tetrahydropyranyl ether, and *tert*-butyldiphenylsilyl group are all removable in acidic media, but the latter is more sensitive than the others, so it can be removed under mild conditions, without attacking of substrate protecting group.

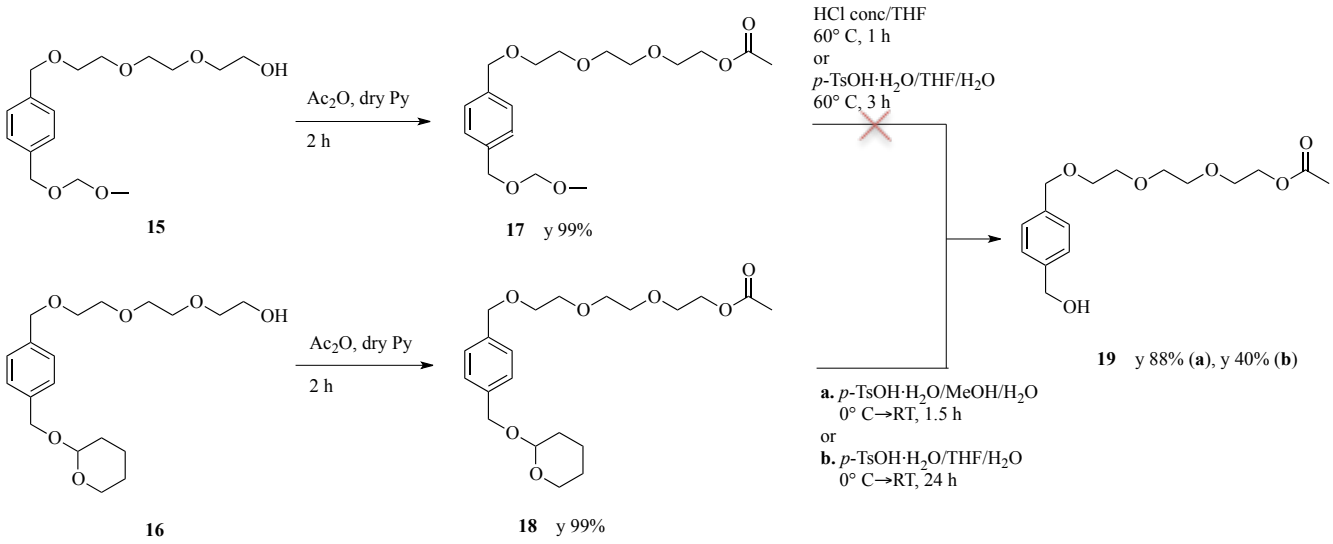
In Scheme 7 are reported the Williamson ether formations between protected substrates **12** and **13,** and the protected or not protected (**6**) PEG chain. There's also shown the removal of *tert*-butyldiphenylsilyl group.



Scheme 7

As shown, all Williamson reactions proceeded well, but removal of *tert*-butyldiphenylsilyl group was impossible. Suiting the silica gel in toluene method, [62] deprotection did not occur; on the other hand, employing TFA conditions, after 1 h only a 50% of compound **14** was deprotected without removal of the acetal group, and after quenching of the reaction and purification by silica gel column chromatography, a complete degradation to a complex mixture was observed.

In Scheme 8 are represented the two parallel ways by which the terminal free PEG hydroxy group in compound **15** and **16** was protected by a species orthogonal both to the acetal and the tetrahydropyranyl ether, that is acetoxy group, removable in basic conditions. Acetoxy group should be resistant to the removal of acetal or tetrahydropyranyl ether, necessary for the subsequent introduction of the azido functionality.



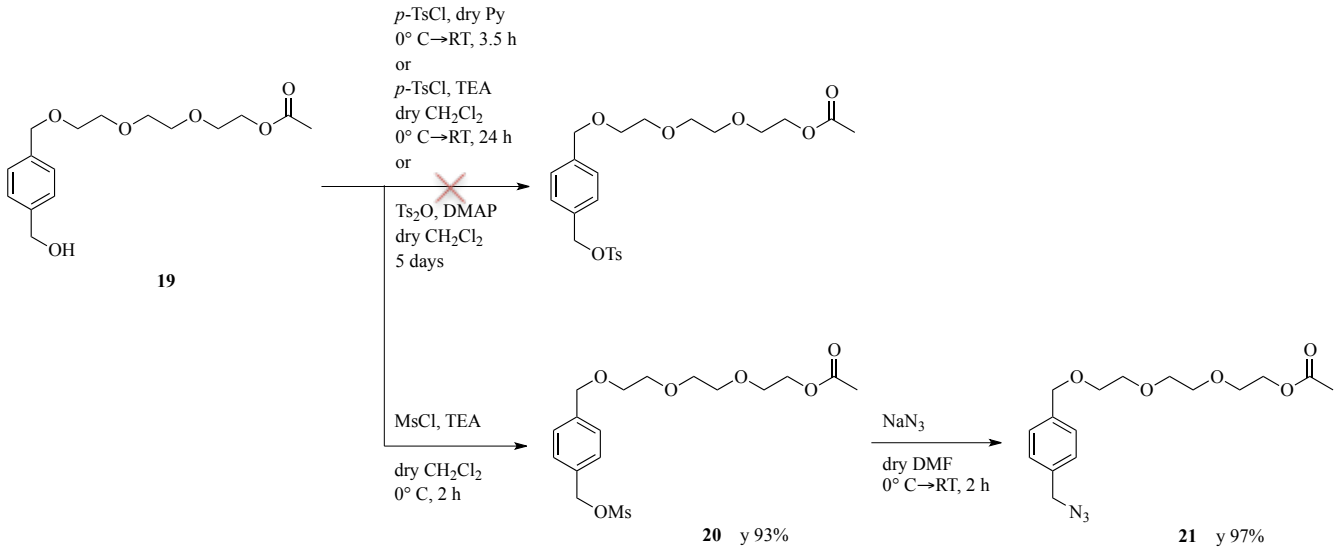
Scheme 8

As shown, acetylation brought in each case to good yields of the protected species. However, removal of acetal group did not give the desired product **19**. [63] In particular, at room temperature **17** was recovered unchanged, but by increasing the temperature a complex mixture of protected, deprotected and degraded products was formed. At the opposite, removal of tetrahydropyranyl ether on compound **18** afforded good yields, especially by using methanol as co-solvent (88%). [64]

The synthetic pathway was so reduced to a unique feasible route, *via* tetrahydropyranyl ether, on which all the following work was consequently based.

I have now to specify that, simultaneously of the described procedure, we undertook an alternative synthetic pathway, that was employing enzymatic catalyzed selective transformations. In order to preserve the flow of discussion, I'll talk about that at last of the current session.

In Scheme 9 are represented the transformations of compound **19** into the corresponding tosylate and mesylate (**20**), and the subsequent introduction of the azido function, by using mesylate as leaving group.



Scheme 9

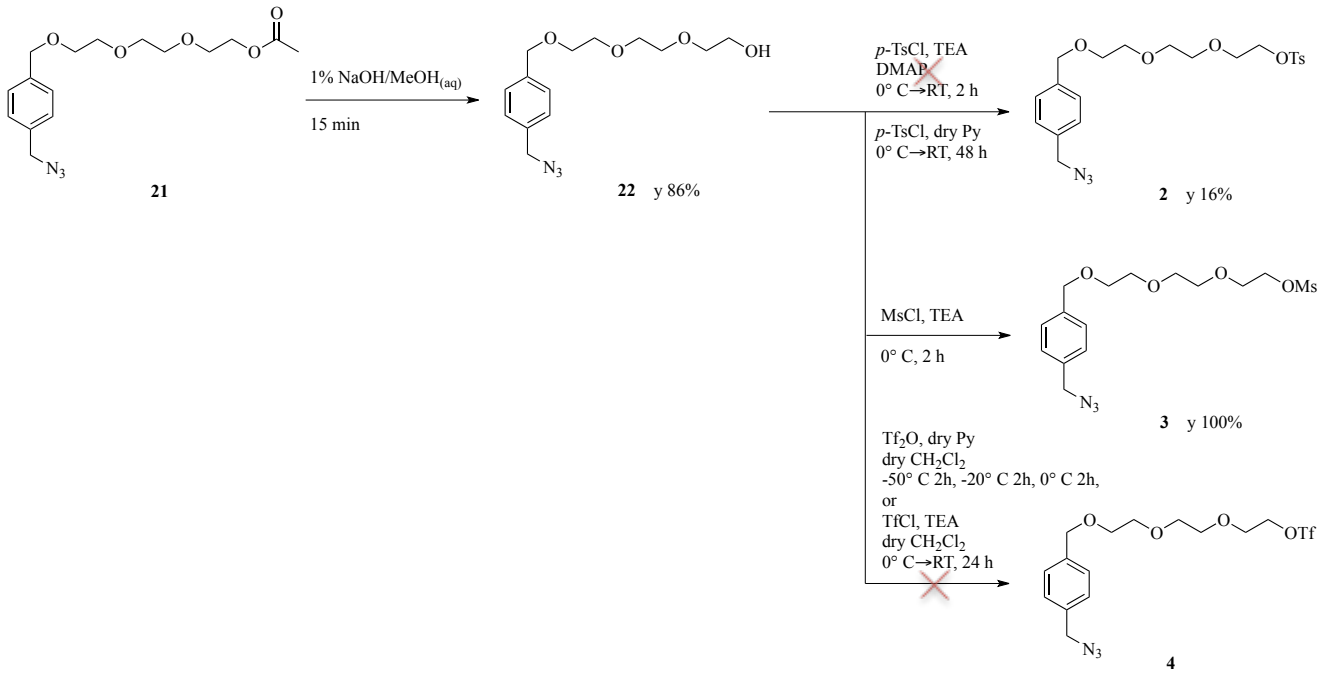
In particular, employing pyridine as base, the desired tosylate was not recovered. For this reason, we tried to set up the same reaction employing triethylamine as base, in anhydrous dichloromethane as solvent. What we found out was that the formed product was the chlorine-derivative. We also noted the presence of tosyl anhydride in the crude product. This result can be explained by a replacement mechanism of the tosylate intermediate by a chlorine atom, assisted by triethylamine, as described in literature. (65)

Thus, we tried to insert the tosylate group by employing tosyl anhydride in presence of dimethylaminopyridine, (66) but what we obtained was only a poor conversion even after five days.

Sorrowful by the experience above, we tried to transform compound **19** in the corresponding mesylate, and the obtained high yield and especially the stability of the resulting product **20**, even in solution at 40° C, was so pleasant as unexpected.

The subsequent introduction of azido group by means of sodium azide in dimethylformamide proceeded without problems bringing to compound **21** in high yields (97%). (41)

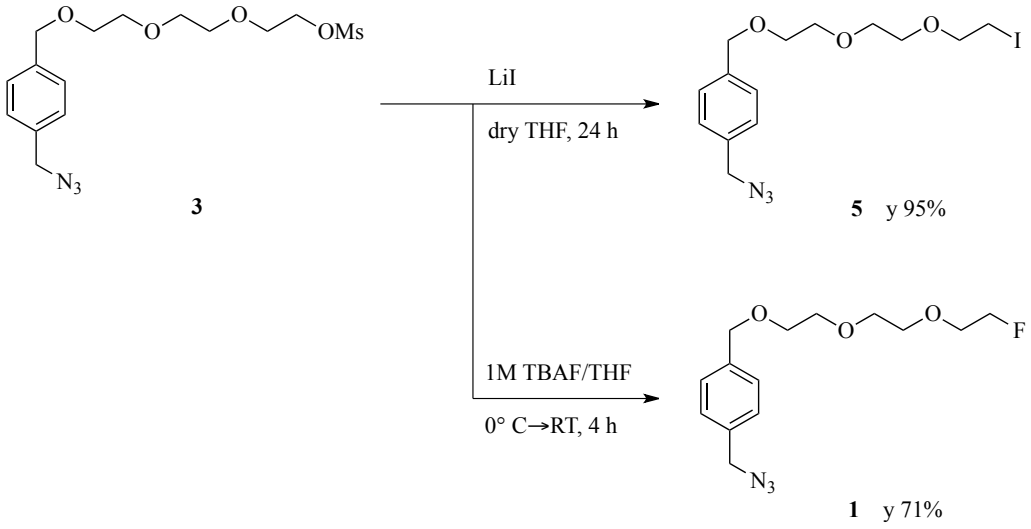
In Scheme 10 is reported the removal of acetyl group from **21**, which provided the starting molecule for the synthesis of possible precursors useful for ^{18}F-radiolabelling: the tosylate-, mesylate-, triflate-, and iodinate-derivative.



Scheme 10

Only a 16% yield was obtained for the tosylate-derivative **2** by the classic method in pyridine, while alternative attempts were not effective. [67] On the other hand, the triflate-derivative **4** preparation was completely unsatisfactory: employing triflic anhydride and pyridine, [68] after formation of a few product, PEG seemed to be somewhat degraded, and, employing triflic chloride and triethylamine, the reaction was too slow and brought to unclear by-products.
On the contrary, mesylate product **3** revealed to be very easy to obtain. Moreover, this product revealed to be very stable.

Starting from mesylated substrate **3**, we prepared the last desired precursor (the iodinate compound **5**), and fluorinated compound **1**, useful as HPLC reference for ^{18}F-radiolabelling monitoring. In Scheme 11 are represented these reactions, that were carried out in good yields.



Scheme 11

In particular, fluorinated compound **1** was obtained by means of mesylate compound **3**, employing tetrabutylammonium fluoride in tetrahydrofuran. [69] From the same substrate **3** we prepared also the iodinate derivative **5**, [70] that, as I will describe later in this dissertation, finally resulted to be the better precursor for ^{18}F-radiolabelling.

1.2 ENZYMATIC APPROACH

As previously mentioned, we started to investigate an alternative synthetic pathway that in some cases was demonstrated to outperform classical chemical synthesis. This was *via* selective transformation of functional groups in a substrate by enzymes.

Enzymes are proteins or RNAs that catalyze biological reactions. The catalytic activity of enzymes proceeds through the formation of an enzyme-substrate complex (ES), in which the substrate binds to a specific region of the enzyme, the active site. Here, the substrate is converted into the reaction product, through a transition state, and it's finally released from the enzyme (Figure 5).

$$S + E \rightleftharpoons ES \rightleftharpoons P + E$$

Figure 5

The binding of a substrate to the active site of an enzyme is a very specific interaction, the result being the lowering of the needed activation energy for the reaction, by favouring the formation of the transition state. The substrate initially binds to the active site by non-covalent interactions, such

as hydrogen bonds, ionic bonds, and hydrophobic interactions, and here multiple mechanisms can accelerate its conversion to the reaction product.

In this way, considering reactions between two substrates, the binding of two or more substrates to the active site in the proper position and orientation can accelerate this reaction (Figure 6). In particular, the enzyme provides a template upon which the reactants are brought together and properly oriented to favour the formation of the transition state in which they interact. Often it is also observed an altering in the conformation of the substrates, approaching that of the transition state, as a changing in the configuration of the enzyme itself. Many enzymes participate directly in the catalytic process: in such cases, specific amino acid side chains in the active site react with the substrate and form bonds with reaction intermediates. [71]

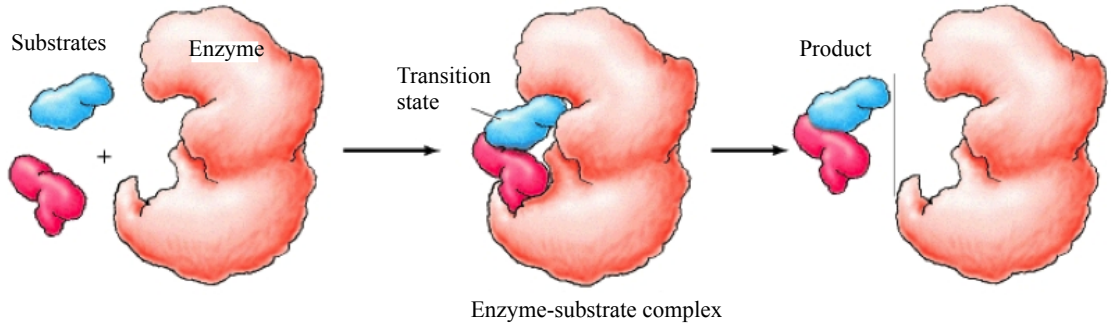
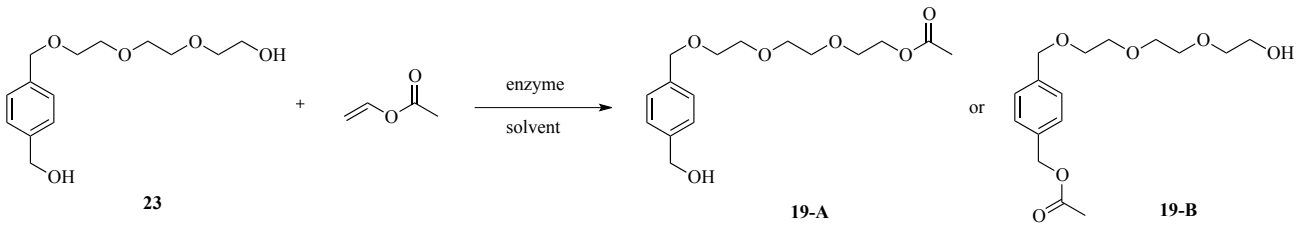


Figure 6

Basing onto this approach, a large variety of reactions were successfully carried out in the last decades, in aqueous as in non-aqueous media. The goal was to simplify current chemical procedures, to built-up clean biocompatible synthesis, and to look towards a more sustainable industry concerning environment respect. Application of enzymatic catalysis as a powerful green enantioselective and chemoselective organocatalytic tool was thus extended to drug development, [72] biofuel production, [73] [74] and architecture control over polymers, complex macromolecules and materials. [75] [76] [77]

Between the wide variety of available enzymes, hydrolases and lipases should be suitable for our purposes, because of their stability (especially for lipases) even in the organic media, and their possibility to catalyze substrate transformations both in esterification and in alcoholysis conditions.

Assays of chemoselective acetylation or deacetylation of substrate **23**, by distinguishing between two different chemical environments, i.e. primary alkyl and benzyl positions, were carried out. In Tables 2 and 3 are represented assays of enzymatic mono-acetylation (Scheme 12) and mono-deacetylation in alcoholysis conditions (Scheme 13).

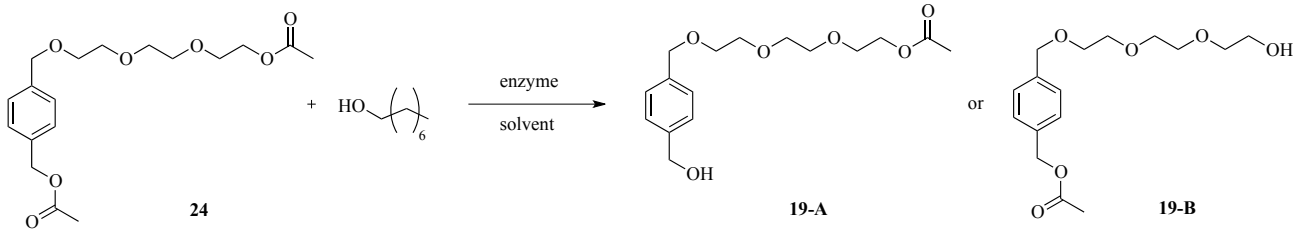


Scheme 12

Enzyme (activity, quantity)	**Vinyl acetate**	**Co-solvent**	**Time**	**23**	**19-A**	**19-B**	**24**
PFL (40,2 U/mg, 2mg)	4 ml	-	28 h	X	X	XX	-
CAL B (5 U/mg, 60mg) [78]	4 ml	-	3 h	-	-	-	X
CCL (5,2 U/mg, 1,7g)	4 ml	-	2 h	-	-	-	X
PPL (23,9 U/mg, 368mg)	4 ml	-	2 h	-	-	-	X
PFL (40,2 U/mg, 2mg) [79]	8 eq	4 ml dry $CHCl_3$	6 h	X	-	-	-
PFL (40,2 U/mg, 2mg)	8 eq	4 ml dry THF	48 h	X	X	X	X
PFL (40,2 U/mg, 2mg)	4 eq	4 ml dry THF	29 h	X	XX	X	-

XX: major obtain product; X: obtained product; -: no product
PFL: Pseudomonas Fluorescence Lipase
CALB: Candida Antarctica Lipase B
CCL: Candida Cylindracea
PPL: Porcine Pancreatic Lipase

Table 2



Scheme 13

Enzyme (activity, quantity)	1-octanol	Co-solvent	Time	24	19-A	19-B	23
PFL (40,2 U/mg, 2mg)	12,4 eq	6ml dry $CHCl_3$	32 h	X	-	-	-
PFL (40,2 U/mg, 2mg)	12,4 eq	6ml dry THF	80 h	-	-	-	X
CAL B (5 U/mg, 93mg)	12,4 eq	6ml dry THF	2.5 h	2,2	0,1	0,9	-

XX: major obtain product; X: obtained product; -: no product
PFL: Pseudomonas Fluorescence Lipase
CALB: Candida Antarctica Lipase B

Table 3

As shown, the only promising way was the employment in alcoholysis conditions of CALB in anhydrous tetrahydrofuran. This result represented our starting point for a complete investigation on enzymatic catalysis, concerning our substrates, the most important assay to be tested being the employment of CALB in anhydrous tetrahydrofuran under mono-acetylating conditions. Any other mixture of the enzyme in vinyl acetate or 1-octanol accelerated the relative conversion in a too fast manner, even by decreasing the enzyme amount.
Even if the mono-acetylated compound 19-B was obtained in promising yields, this study was finally set apart. Anyway, this result could constitute the starting point for an eventual future investigation.

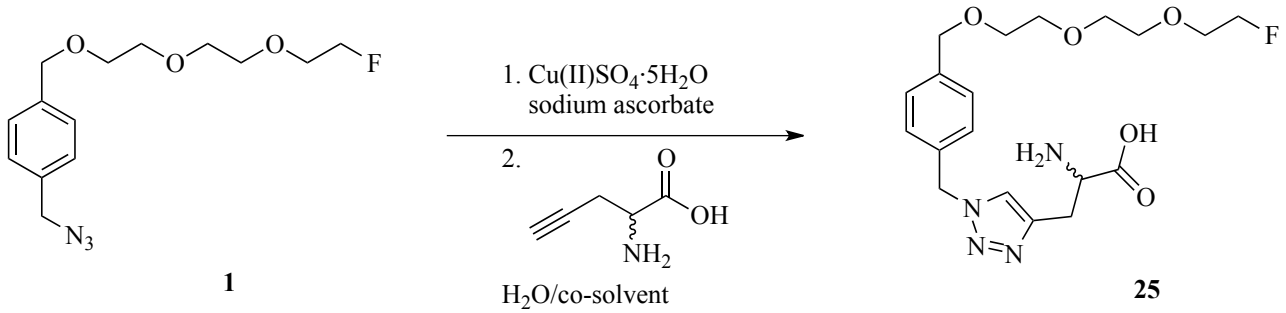
1.3 CuAAC Study

As we successfully obtained compound **1**, we could proceed in the development of the [3+2]Huisgen dipolar cycloaddition between **1** and a cost-effective simple alkynyl-modified molecule as model of choice, that is commercial non-protected D,L-propargylglycine.
To this regard, I previously described the reasons that brought us to directly employing CuAAC using $Cu(II)SO_4 \cdot 5H_2O$ *in situ* reduction by an excess of sodium ascorbate. The reaction conditions we initially selected were similar to Glaser and Årstad described ones, [16] that are by employing a mixture of water and *N*,*N*-dimethylformamide as co-solvent, and classic chemical work-up of the reaction mixture. The low amount of fluorine substrate we employed, the low yield obtained by the

authors (25%), and the supposed high hydrophilicity of our product lead us not to purify product **25** by column chromatography on silica gel, but to analyse and purify it by RP-HPLC. The identity of the obtained desired product was confirmed by ESI-MS.

Our efforts were thus concentrated on the improvement and optimization of such a synthetic procedure, in order to investigate the best conditions to be used in the corresponding radiochemical assays. To this regard, a series of tests were carried out by varying reaction media, concentration and amount of reagents, quenching agents, and purification procedures.

We immediately noted that a too big amount of copper catalyst and sodium ascorbate caused significant precipitation from the reaction mixture. In order to avoid this, we used almost five-fold diluted catalyst compared to Glaser and Årstad recommendation. In Tables 4 and 5 are summarized CuAAC assays (Scheme 14).



Scheme 14

Reagent	Quantity	Eq	Reagent solution concentration	Starting reaction concentration
$Cu(II)SO_4 \cdot 5H_2O$	1.5-15 mg	1.8	0.12 - 0.14 M	15 - 31 mM
Sodium ascorbate	3.6-36 mg	5.4	0.40 - 0.74 M	45 - 92 mM
Propargylglycine	0.5-5 mg	1.2	0.03 - 0.08 M	10 - 21 mM
Fluoroazide 1	1-10 mg	1	0.02 - 0.03 M	8 - 17 mM

Table 4

Water (% volume)	Co-solvent (% volume)	Reaction volume	Time for 100% conversion
25%	75% DMF	2 ml	2 h
60%	40% CH_3CN	0.4-0.8 ml → concentrated	1.5 h
60%	40% CH_3CN	0.8 ml	> 5 h
60%	25% CH_3CN + 10% DMSO	0.8 ml → concentrated	2 h
70%	20% CH_3CN + 10% DMSO	1 ml	3 h
70%	30% EtOH	1 ml	> 2 h
70%	30% EtOH	1 ml → concentrated	1 h

Table 5

What we definitely found out was that the use of acetonitrile slowed down the cycloaddition rate, but it avoided the strong precipitation from the reaction mixture observed employing *N*,*N*-dimethylformamide or dimethyl sulfoxyde. On the other hand, the reaction carried out in ethanol as co-solvent seemed to be a little more efficient than in acetonitrile, without presenting a too strong precipitation. This was explained by the major stabilizing effect of Cu(I) species especially of acetonitrile over *N*,*N*-dimethylformamide or dimethyl sulfoxyde, that reduces copper aggregates formation and lowers the reaction rate. [80] The compromise seemed to be the use of a small amount of dimethyl sulfoxyde, and the partial removal of solvents (acetonitrile or ethanol) from the starting reaction mixture during the reaction.

Formation of compound **25** was, as previously mentioned, successfully monitored by an optimized RP-HPLC method, as shown in Figure 7.

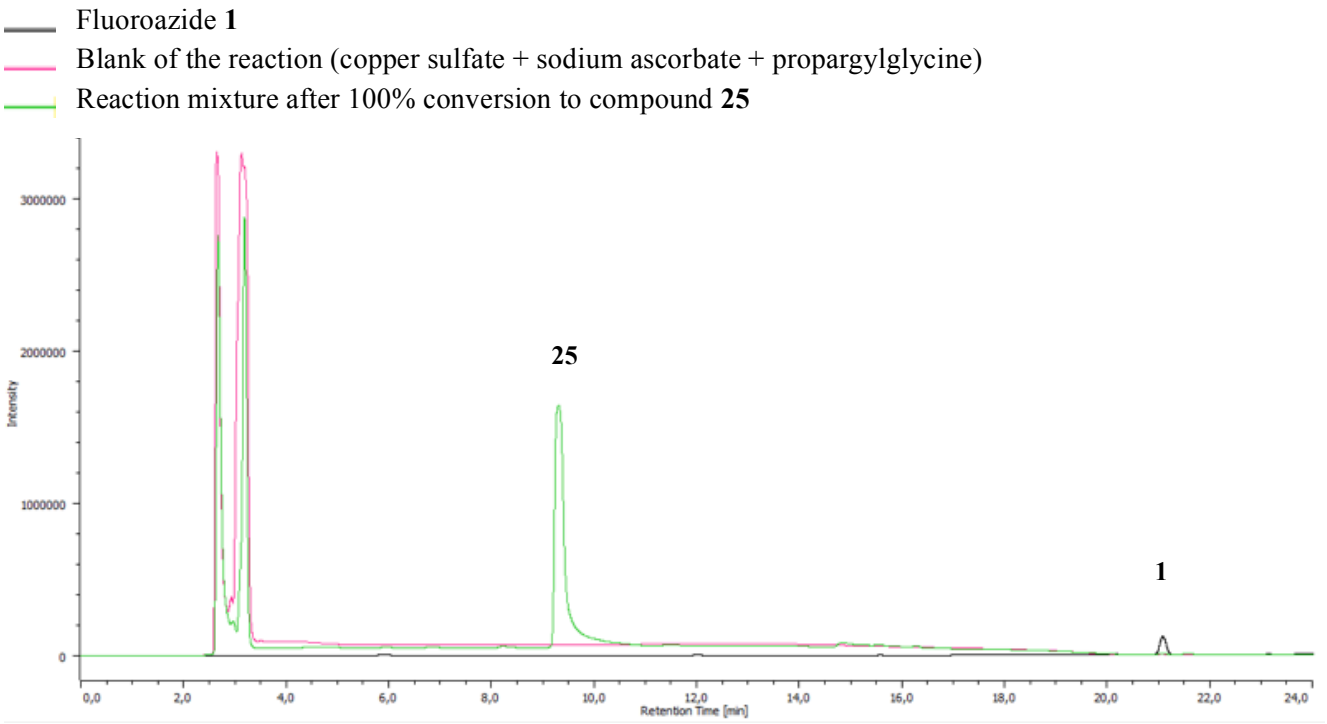


Figure 7

In detail, we used a C18 XTerra analytical column (250x4,6mm 5µm) and a gradient from 90:10 to 30:70 water/acetonitrile in 24 min, with a 1 ml/min flow, observing absorbance at 220 nm.
As shown in the chromatogram, fluoroazide **1** (t_R 21.2 min) disappeared from the reaction mixture and the only formed product was 1,2,3-triazole **25** (t_R 9.4 min).

After completion of CuAAC, a little or great precipitation always occurs, depending on the nature of the used co-solvent, its amount, copper catalyst quantity, and the partial removal of the solvent from the starting reaction mixture. This is due to copper aggregates formation, as I mentioned in the earliest sessions of this thesis.
As the reaction mixture had next to be injected in a semi-preparative HPLC column, either for purification or reaction monitoring purposes, the precipitate had to be removed by dissolution of the final reaction suspension or, alternatively, by mechanic filtration.
Quenching conditions and dissolution outcomes are summarized in table 5.

Reagents	Reagents concentration	Dissolution of aggregates	Collateral effects
HCl	1 M	Small volumes are completely solubilized. Bigger volumes need more time.	No contraindications.
TFA	10%	Small volumes are completely solubilized. Bigger volumes need more time.	No useful separation from the product during semipreparative HPLC.
NaOH	1 M	Small volumes are completely solubilized. Bigger volumes only partially solubilized.	Peak in analytical HPLC is broadened.
TEA	pure	No solubilization.	-
Acetic acid	glacial	Long time solubilization.	-

Table 5

What we found out was that, depending on the used co-solvent, the dissolution or the filtration treatment, 1,2,3-triazole **25** changed retention time in the same analytical RP-HPLC conditions described above, ranging from 8 to 11 min. Also the peak shape seemed to change. For instance, in Figure 8 is reported the injection of a reaction mixture containing dimethyl sulfoxyde as co-solvent and quenched with 1 M hydrochloric acid, where the retention time was in this case 8.6 min, and the peak shape more definite. For comparison, Figure 7 reports the injection of a reaction mixture diluted in acetonitrile (no precipitation was observed at high dilution).

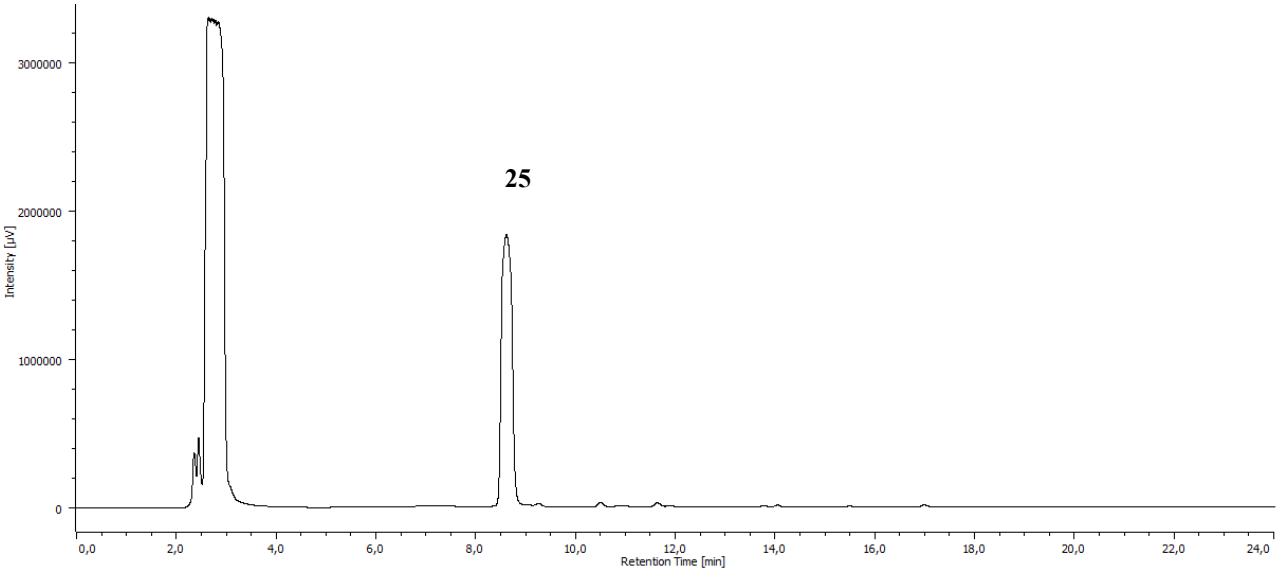


Figure 8

This finding was supposed to be the direct consequence of possible complex formation between the product and copper ions, that also compromised purification of compound **25**, as showed next in this dissertation.

Tests on crude 1,2,3-triazole **25** were performed in order to develop different purification methods. The first method consisted in quenching the reaction mixture with 1 M hydrochloric acid, then loading the obtained solution onto a tC18 Sep-Pak cartridge, washing the cartridge, and finally eluting the desired product with a polar organic solvent. In particular, the best result was obtained by diluting the quenched reaction mixture in a solution of water/acetonitrile 95:5 and by loading it onto the Sep-Pak cartridge, by washing first with water, then with a solution of water/acetonitrile 95:5, and finally by collecting the desired product **25** with a few millilitres of acetonitrile or ethanol. A shown in Figure 9, the desired product was obtained with suitable purity.

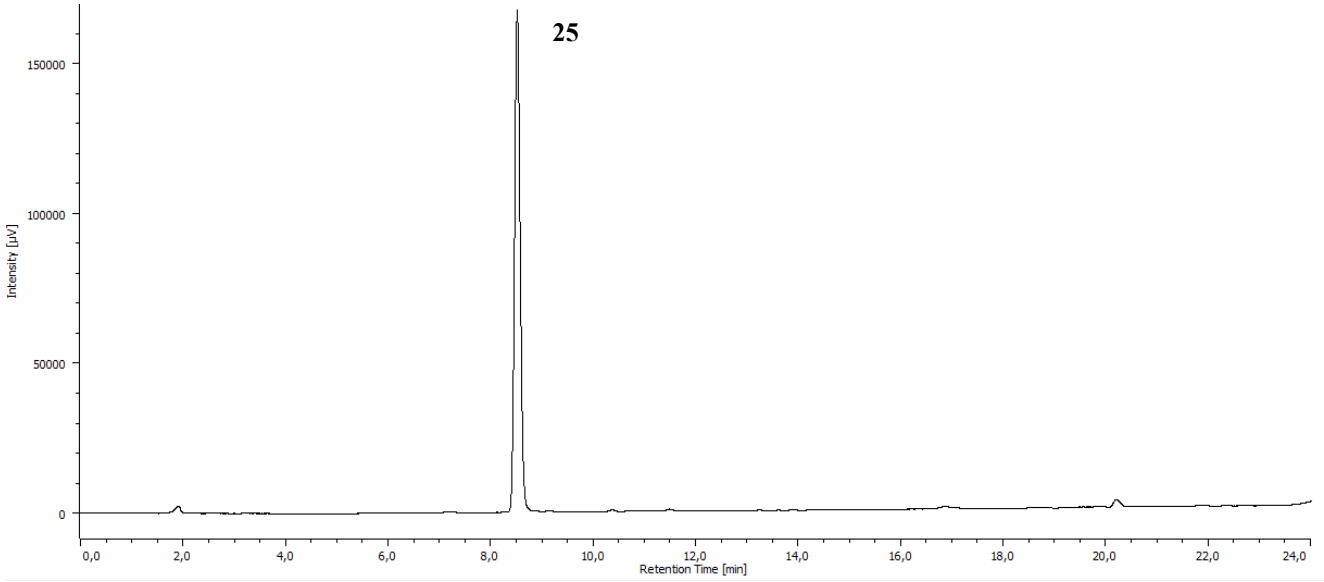


Figure 9

The second purification procedure was the direct filtration of the reaction mixture onto a filter septum that was then washed with a water/acetonitrile 95:5 solution. The filter removed efficiently copper aggregates and a limpid solution was collected. In order to obtain the purified 1,2,3-triazole **25**, an additional step employing a Sep-Pak cartridge was necessary. This was carried out in a similar manner to what described for the first method of purification. However, analysis of the solid residue revealed also the presence of the product. Washing residual powder with 1 M hydrochloric acid before purification of the collected solutions didn't give better results.

Both methods brought only to a 50% final yield regarding 1,2,3-triazole **25**. Moreover, after elution of the product from the Sep-Pak cartridge with a polar organic solvent, the subsequent concentration of the volume provided a white crystalline powder that resulted difficult to be solubilized under all tested conditions. Only a large excess of water or organic media could re-solubilize such a suspension. For these reasons, product characterizations requiring higher concentration of solute, such as ^{13}C-NMR presented some problems.

These findings lead us to hypothesize that hydrochloric acid can only dissolve copper aggregates, but complexes formed between the product and copper ions were still present in the solubilized mixture. These complexes were partially irreversibly bonded to the Sep-Pak resin, and partially eluted and collected in what was the final purified form of 1,2,3-triazole **25**. This theory is supported by the known, documented fact that glycine can form complexes with copper ions. At pH between 4 and 8 the predominant form is the neutral complex formed from one copper and two zwitterionic glycines (Figure 10), and at pH higher than 8 the basic complex was observed. Complex formation progressively diminishes with suppression of ionization of the carboxyl group

and disappears at high acidity, so at pH lower than 4 only the zwitterion ion glycine participates in this equilibrium. At pH 6 in solution of diluted amino acid it was found the particular formation of Cu_2-$Glycine_3$, as acidic complex. [81] [82]

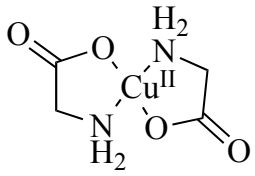


Figure 10

It appears realistic that also the triazole moiety can undertake salt formation with a copper ion. The σ-basicity character of nitrogen in position 3, and the π-acidity of the ring, can stabilize Cu^{++} ions, as just demonstrated for imidazole ring (and for histidine), [83] but also Cu^{+} ions. [84]

The proximity of triazolic N_3 and amino group of glycine in our product **25**, can ideally bring to the formation of a complex involving both these groups in a 6-membered ring, to achieve a bis-1,2,3-triazole **25** complex of Cu(II), as hypothesized in Figure 11, where the hybrid bis-1,2,3-triazole **25** complex and the glycine-like bis-1,2,3-triazole **25** complex are also shown.

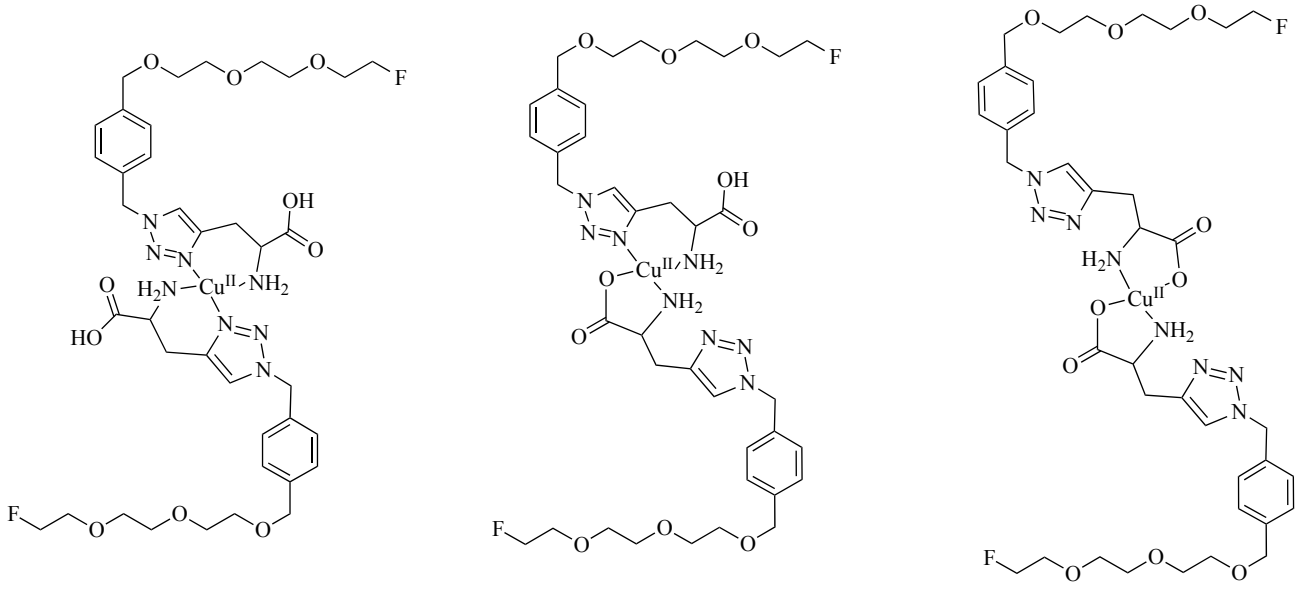


Figure 11

The scenario above is further complicated by the fact that also chlorine atoms, released by hydrochloric acid, in strong acidic conditions can participate as ligand for copper complexes containing the triazole product. The increasing of the amount of hydrochloric acid can sequestrate copper by forming $CuCl_2$ salt, but this reaction can require more time because Cu(II) can be involved in a redox equilibrium with Cu(I) even in strong acidic conditions. [85] is probably the

reason by which, after treatment of our reaction mixture with an excess of 1 M hydrochloric acid compared to copper catalyst, the complete dissolution can take more than 15 min, and it is strongly slowed down if the mixture was diluted.

The same problem of a final low product yield was definitely found also in a parallel purification method, developed for final purification of ^{18}F-radiolabelled triazole product, where the HPLC column stationary phase seemed to interact with copper complexes, in analogy with purification by Sep-Pak cartridges, as it will be described in the next chapter of this document.

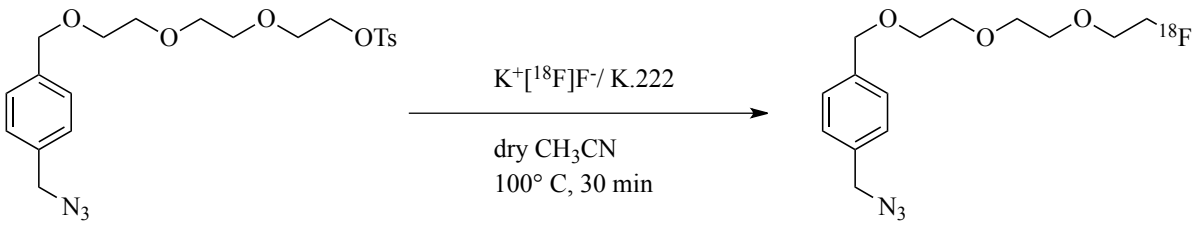
Thus, it's clear that more effective solutions have to be found to remove copper species from 1,2,3-triazole **25**. This could be possible using a more concentrate quenching solution (for example 2-4 M hydrochloric acid or sodium hydroxide), or employing a chelating agent such as ethylenediaminetetraacetic acid (EDTA), the latter taking advantage from the fact that the equilibrium generally moves towards the formation of a chelate than a complex.
Concerning other biomolecules different than glycine, it will be possible that quenching the reaction mixture will not be necessary if copper complexation not occurs. However, a documented interaction of other aminoacids with copper ions indicates that this problem may persist, so the use of EDTA shall be an interesting solution to perform, to avoid excessive low or high pH that can damage labile biomolecule functionalities. [86]

1. RADIOCHEMICAL SESSION

All radiochemical synthesis, analysis, and purifications discussed in this chapter were performed in the radiochemical laboratories of University of MILANO-BICOCCA (Tecnomed Foundation).

In order to afford the desired labelled azide **[^{18}F]1**, various experiment were performed. All procedures were carried out onto a fully automated system for F-18 radiolabelling.
F-18 was produced on-site following standard protocols for an 18 MeV proton beam cyclotron. At the end of the bombardment (EOB) it was transferred to the synthesis module in the form of [^{18}F]fluoride ion in aqueous solution and trapped onto an anionic exchange cartridge, from which was eluted with aqueous potassium carbonate as carbonate salt. A cryptand (Kryptofix.222) dissolved in acetonitrile was added, and the mixture was azeotropically distilled to provide anhydrous $K^+[^{18}F]F^-$/K.222 salt, ready to be used in the S_N2 reaction.

Our investigation started considering tosylate azide **2** as useful precursor to achieve **[^{18}F]1**. Various experiments were carried out by varying the temperature, but without reliable results. The finally optimized procedure resulted to be the S_N2 reaction in anhydrous acetonitrile, at 100° C, with final removal of unreacted [^{18}F]fluorine by passing the reaction mixture through Sep-Pak Al light N cartridges (Scheme 15).



2 **[^{18}F]1** rcy 1%

Scheme 15

Analysis by RP-HPLC using an XTerra C18 column, with a water/acetonitrile gradient from 60:40 to 20:80 in 20 min, at 1 ml/min flow, monitoring at 220 nm (Figure 12a) and by a radiochemical detector (Figure 12b), revealed that the product **[^{18}F]1** was formed only with a 1% RCY (radiochemical yield) non-corrected for the decay (52% radiochemical purity).

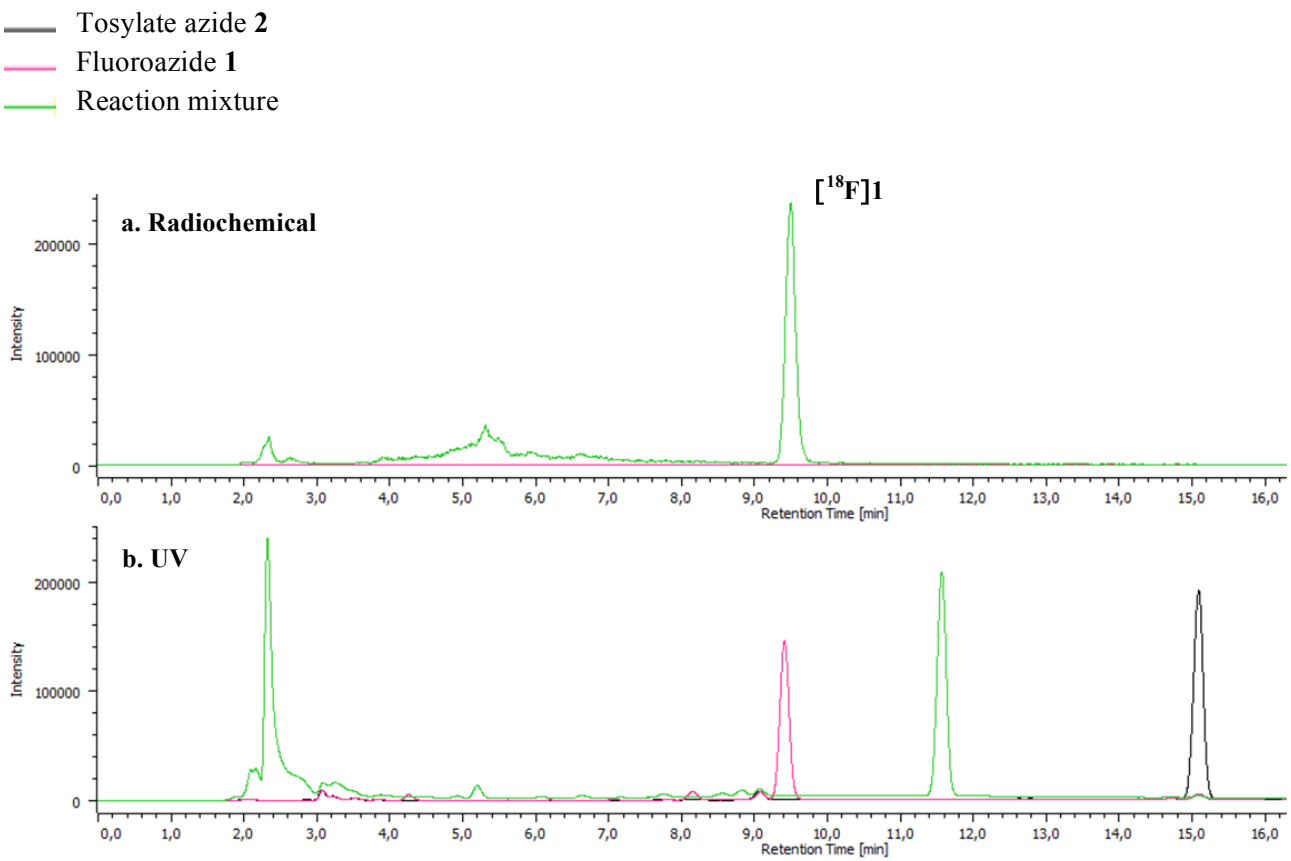
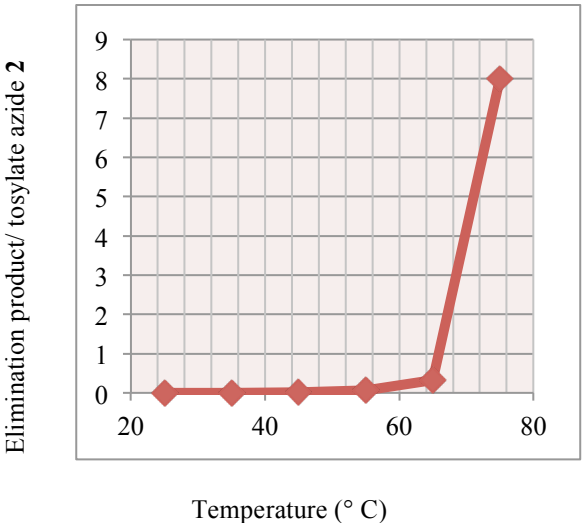


Figure 12

The UV spectra showed the complete degradation of the precursor to afford the elimination product (t_R 11.5-12.5 min), as confirmed later by LC-MS analysis of the reaction mixture.

This very low yield couldn't be improved, and the main species observed by RP-HPLC was unreacted F-18 that, bounded to the kryptand, was eluted in the early minutes from the column (retention times for Kryptofix were confirmed by ESI-MS, those corresponding to radiochemical obtained signals).

Thus, in order to better understand precursor stability in reaction conditions, tests were carried out, demonstrating that it undergoes significant degradation from the temperature of 70° C (Table 6).

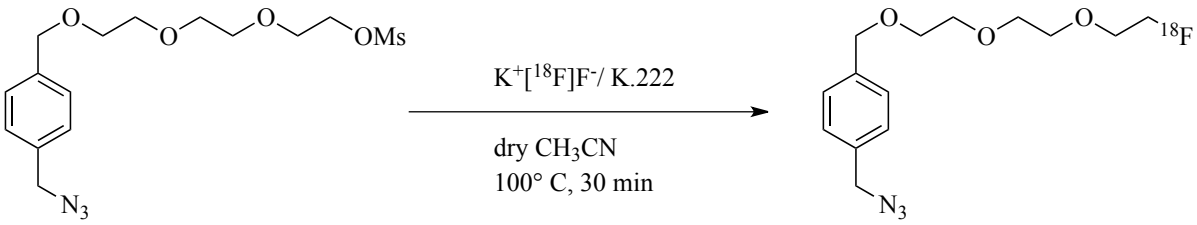


t (°C)	Elimination product/tosylate azide 2
25	0,00
35	0,00
45	0,02
55	0,07
65	0,33
75	8,00

Table 6

Because of this very low obtained yield, we decided to proceed in our inspection considering mesylate precursor **3**. Radiolabelling tests at 30, 70, and 100° C were performed under the same conditions used for precursor **2**, the same final purification by Sep-Pak Al light N cartridges included, and employing an analogue monitoring RP-HPLC method.

For reactions carried out at 30° C and 50° C only a few radiolabelled product **[^{18}F]1** was observed, even if in both cases better results were obtained than for tosylate precursor **1**. It was at 100° C that a really good RCY was obtained, that was 57% non-corrected for the decay (74% radiochemical purity).



3 **[^{18}F]1** rcy 57%

Scheme 16

This was surely a great result, but especially observing the UV plot some perplexities arose immediately. First of all, the chromatogram appeared too complex and the possibility to purify the radiolabelled product **[^{18}F]1** just by Sep-Pak cartridges seemed to be unreliable. As purification of such a reaction mixture is mandatory, especially because azido species may affect the subsequent CuAAC step by competing with **[^{18}F]1**, purification could be achieved only by semipreparative HPLC. This could represent a problem if also purification of the final CuAAC radiolabelled product by semipreparative HPLC should be required, because the long time necessary to complete the

whole procedure, but especially because not feasible in practice, as our synthesis module is equipped with instrumentation useful for a lonely HPLC purification per process. Moreover, under these reaction conditions the mesylate precursor **3** partially degrades to the hydrolysis product (t_R 5-6 min, as demonstrated by co-injection of alcohol azide **22**) and elimination product (t_R 11.5-12.5 min). Considering retention times for mesylate precursor **3** and the radiolabelled product are very close one another, also semipreparative HPLC purification of the radiolabelled product from precursor should be difficult to achieve (Figure 13).

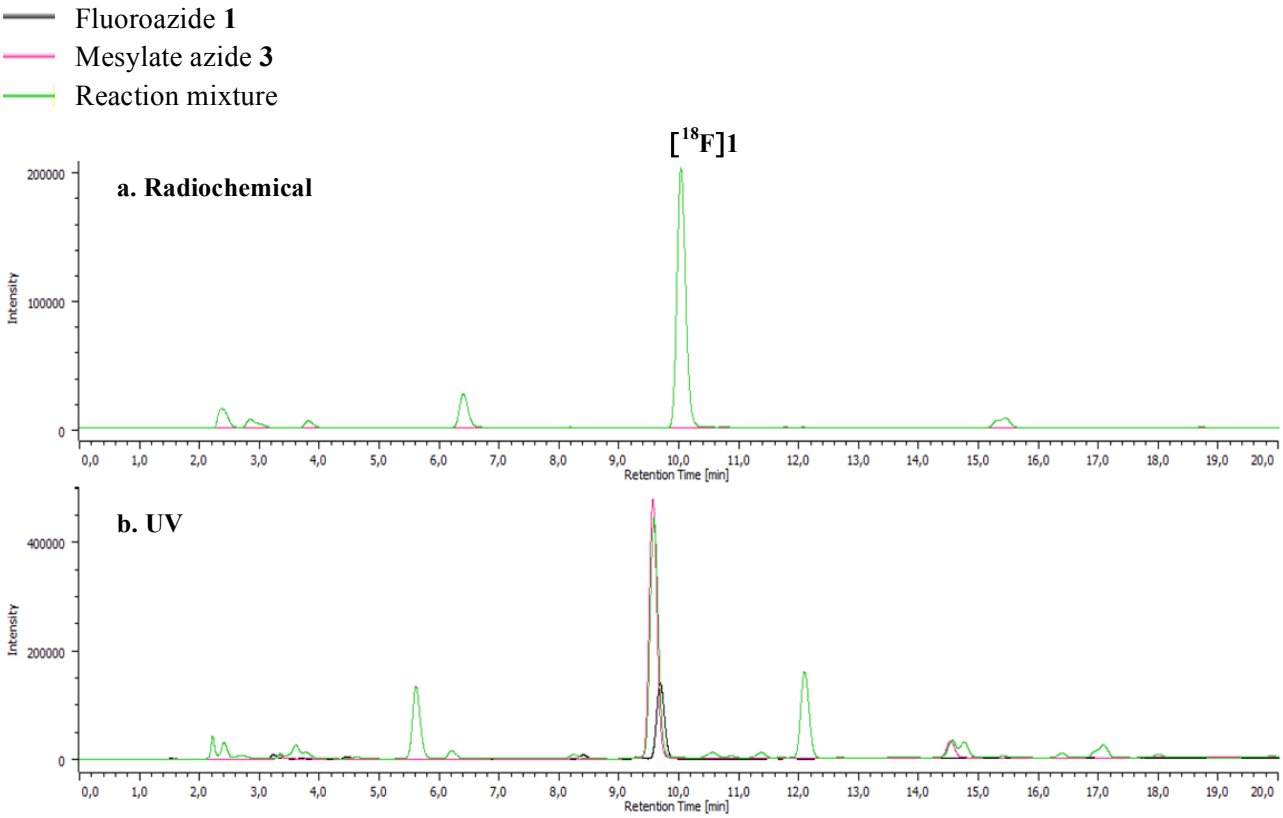


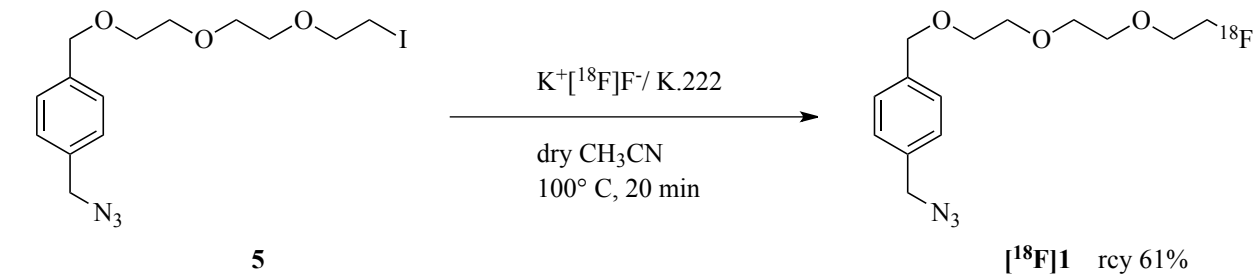
Figure 13

As the reaction proceeded in high yields, it appears obvious that degradation products were formed only after quenching the reaction with a water media. On the other hand, the chromatogram in figure 13 reflects the real composition of the reaction mixture dissolved in water, before purification.

Standing on these results, we proceeded to investigate the utility of iodinate precursor **5**.

From radiolabelling tests it was immediately clear that precursor **5** was definitely the most promising one. The obtained radiochemical yield for **[^{18}F]1** (not corrected for the decay) was 61%

(85% radiochemical purity) after 20 minutes, thus a better result than employing precursor **3** (Scheme 17).



5 **[^{18}F]1** rcy 61%

Scheme 17

The more interesting result was the very clean and definite radiochemical and UV plots (Figure 14):

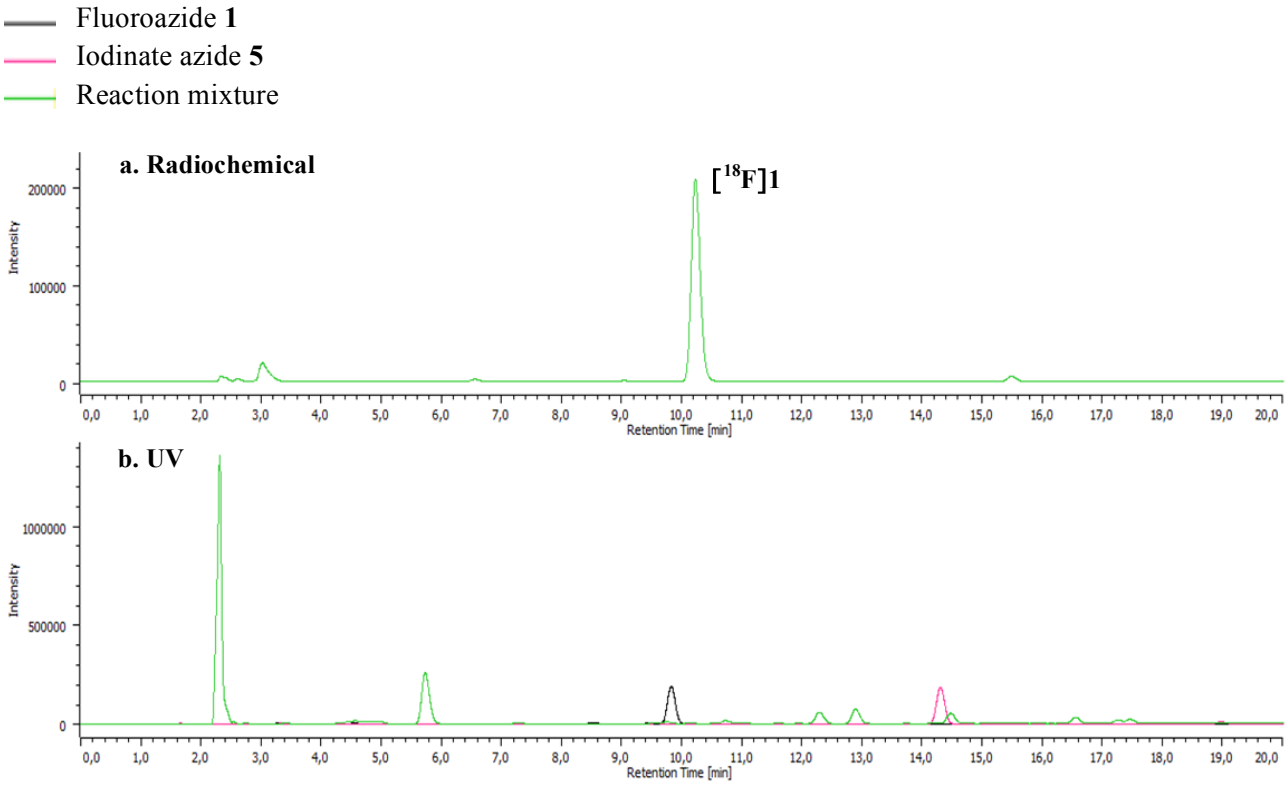


Figure 14

As shown, iodinate precursor **5** degraded completely during radiolabelling reaction to finally achieve free iodine (solvent front) and alcohol azide (t_R 5-6 min).

Stability tests of precursor **5** were performed, to better understand degradation mechanism.

Concerning temperature stability, precursor **5** was heated to 100° C for 20 minutes in anhydrous acetonitrile, and monitored after intervals of 5 minutes. The precursor resulted totally stable. On the other hand, submitting the precursor to reaction-like conditions, yet at room temperature it revealed

to be completely degraded. As already seen for mesylate precursor **3**, we could suppose that hydrolysis occurs when precursor **5** in a basic environment is diluted in water. However, additional assays in which, in presence of the basic salt and before dilution with water, a PBS buffer was added to stabilize the pH of the solution at a neutral value demonstrated that hydrolysis at a neutral pH occur too. This could only signify that explanation of the hydrolysis process could be attributed to the improvement of water nucleophilicity by the phase transfer. Similar tests were carried out also employing a phase transfer alternative to K.222, such as tetrabutylammonium hydrogen carbonate ($TBAHCO_3$), before or after distillation process. The obtained results showed that the phase transfer improves water nucleophilicity only after distillation, therefore causing precursor hydrolysis, even under pH stabilization at neutrality. In this case, the phase transfer results suspended in pure acetonitrile, and hydrolysis is easily catalyzed simple by adding water.

So that, the only fact yet to be verified was the effective complexity of the resulting UV chromatogram concerning a radiolabelling procedure that employs tetrabutylammonium hydrogen carbonate, and the final radiochemical yield. Unfortunately, as shown in Figure 15, even if the radiochemical yield was too high (57% non-corrected for the decay, 95% radiochemical purity), the plot revealed to be too much complicate to be resolved by Sep-Pak cartridges purification, compared to the analogue obtained from radiolabelling with kryptofix.222, so this way was definitively set apart.

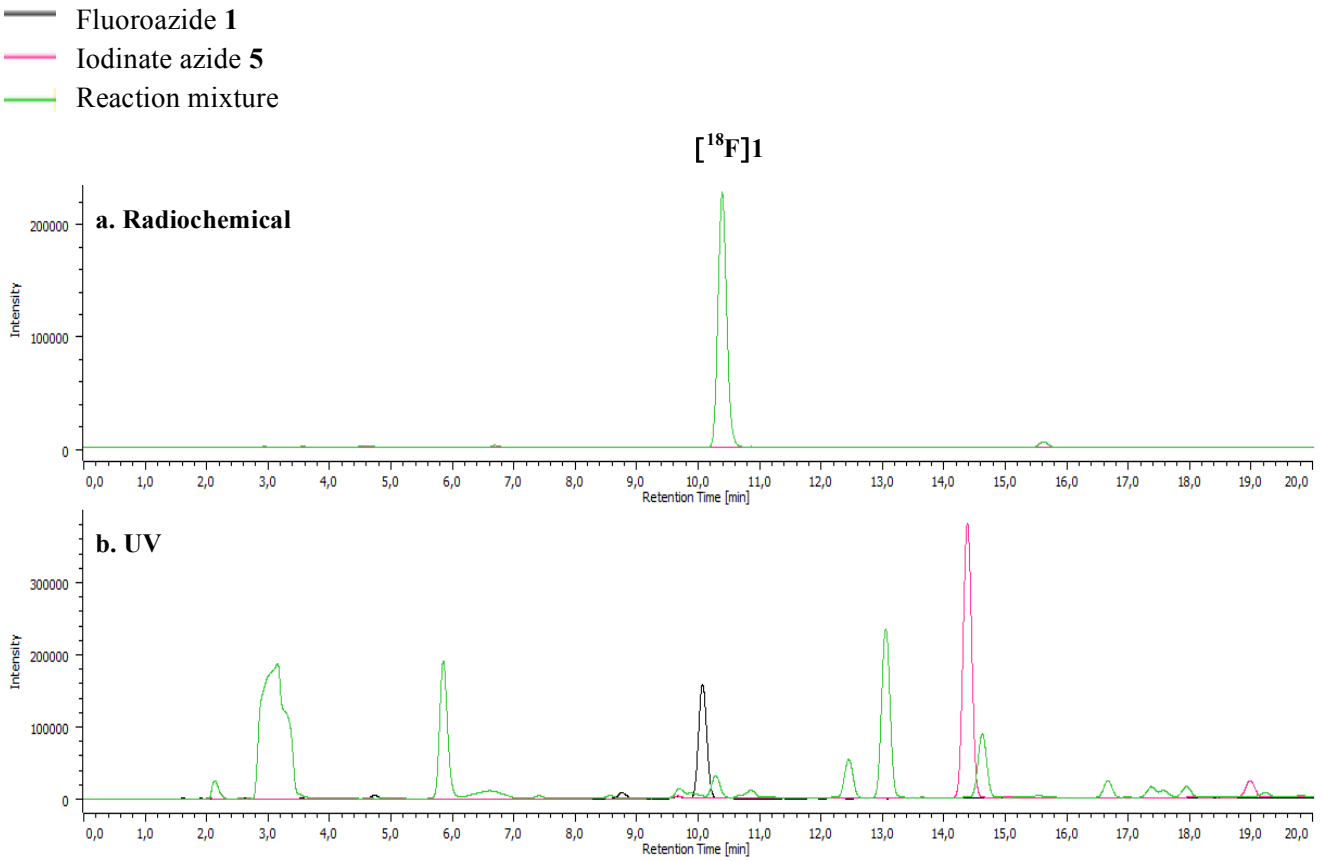


Figure 15

Coming back to the more promising route, that was production of **[^{18}F]1** *via* radiolabelling of iodinate precursor **5**, our efforts were concentrated onto purification of the F-18 radiolabelled azide.

The method we optimized employs a Sep-Pak tC18 cartridge. In details, after removal of non-reacted F-18 by Sep-Pak Al light N cartridges, the reaction mixture containing the iodinate precursor **5** and the radiolabelled product **[^{18}F]1** was diluted in a water/acetonitrile 9:1 solution and passed through a tC18 Sep-Pak cartridge. The cartridge was washed with a water/acetonitrile 8:2 solution and the product **[^{18}F]1** was eluted with a few millilitres of acetonitrile or ethanol. Figure 16 shows the successful of this procedure, by which product **[^{18}F]1** was obtained with a radiochemical yield of 51% (non-corrected for the decay, 93% radiochemical purity).

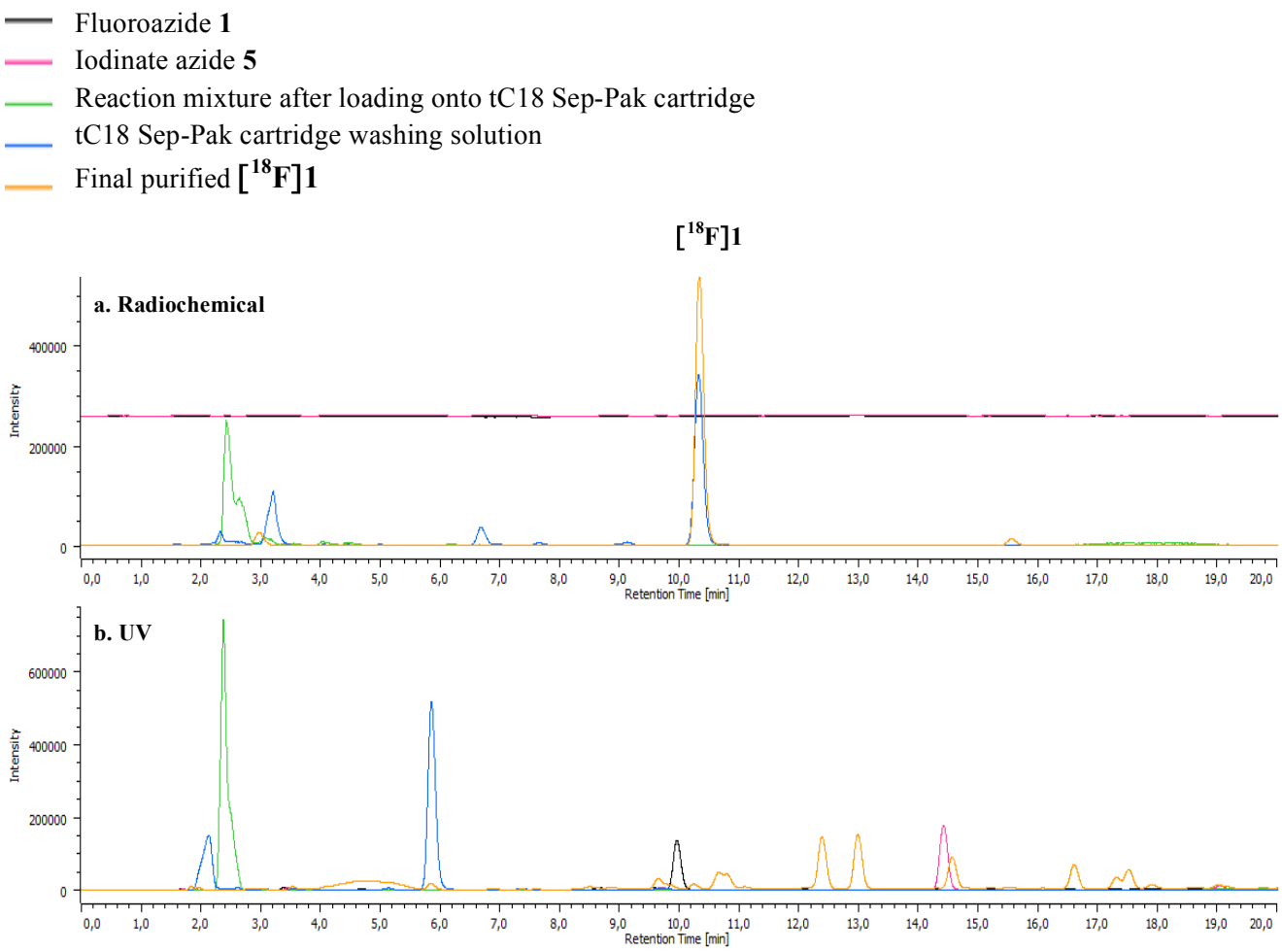
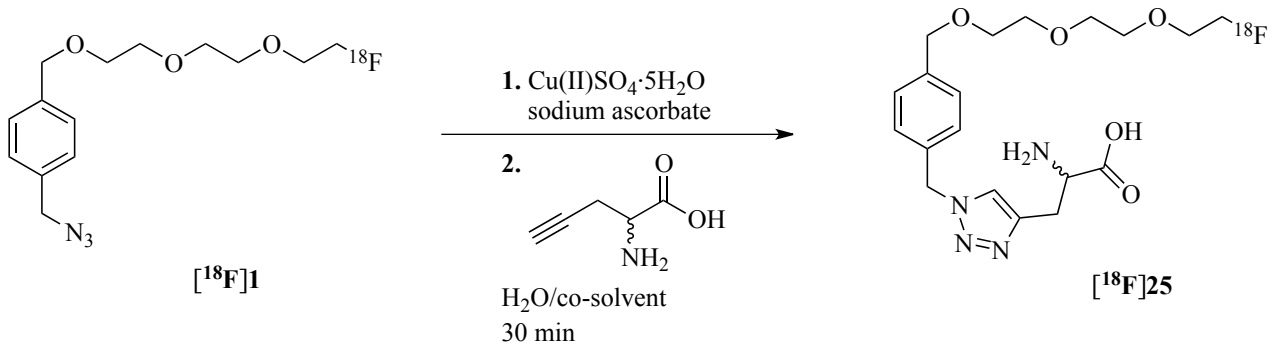


Figure 16

As shown, UV profile seemed to be sufficiently free from by-products, so that we could dedicate to the following step, that is radiochemical CuAAC employing the purified product **[^{18}F]1**.

As mentioned before, we decided to test a simple, cost-effective alkynyl-modified amino acid, whose functionalities are unprotected. This should avoid the deprotection final step, and the eventual presence of by-products and residual starting materials. Sure, also protected biomolecules can be used too, and normally this is the most frequent situation in radiochemistry, but in general, as radiopharmaceuticals are not necessary required in high amounts to be employed in a clinic or pre-clinic assay, but only in high chemical and especially radiochemical purities, we judged better to focus ourselves on the development of a general purpose tool for the "click" labelling with F-18, and avoid more complex pathways.

The radiolabelling CuAAC we tested is reported in Scheme 18, in similar conditions to cold analogue synthesis. [16] This was carried out using the ^{18}F-radiolabelled azide purified by the previously reported Sep-Pak method, and an acetonitrile/dimethyl sulfoxide mixture as co-solvent.



Scheme 18

After quenching by 1 M hydrochloric acid, the reaction mixture was diluted with water and monitored by analytical RP-HPLC, employing the same column XTerra cited before, using the gradient method from 90:10 to 30:70 water/acetonitrile in 24min, with 1ml/min flow. The formation of the product was observed by radiochemical detector (Figure 17a), and by UV detector at 220nm (Figure 17b).

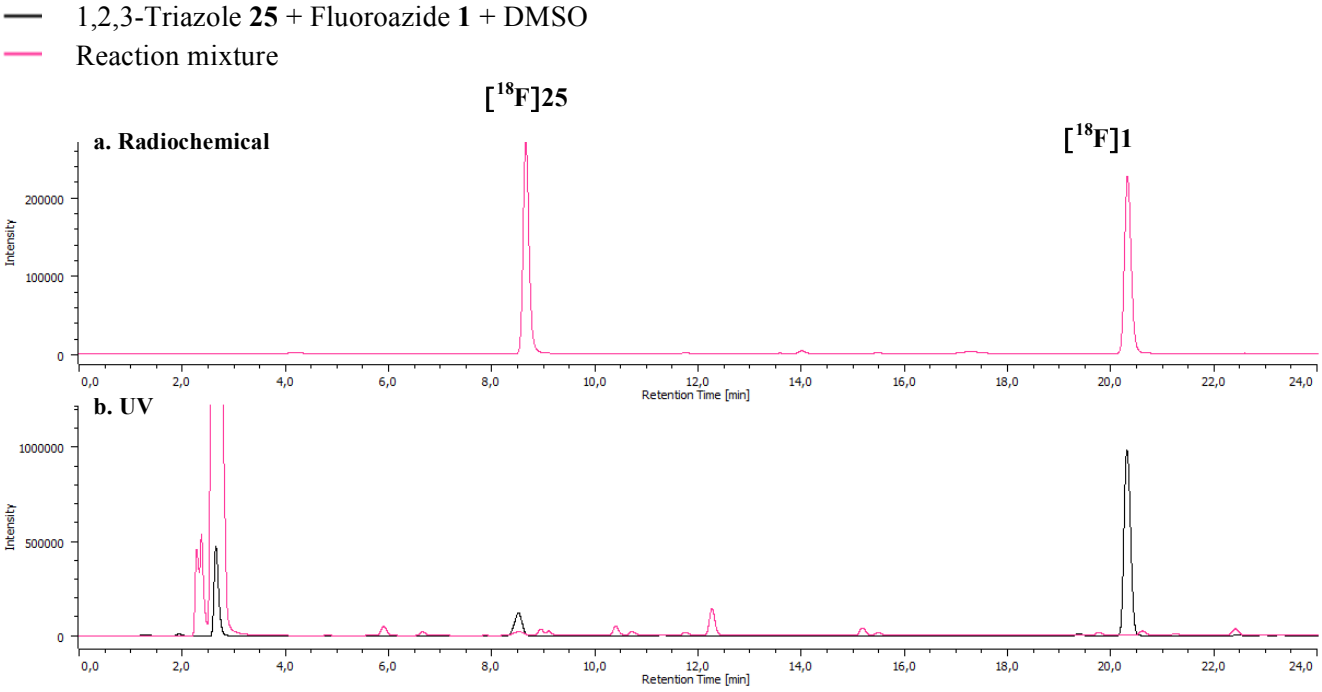


Figure 17

As shown, in this conditions we could obtain a 52% conversion of **[^{18}F]1** (t_R 20.3 min) to give **[^{18}F]25** (t_R 8.7 min).

The calculated radiochemical yield resulted to be 18% non-corrected for the decay.

Standing on this positive result, the following step consisted in purification of crude **[^{18}F]25**.

To this regard, a semipreparative RP-HPLC method employing 0.1% TFA as buffer was developed, using an XBridge PREP C18 (10x150 mm, 5 µm) column. The method was: isocratic 0.5 min 90:10 water (+0,1% TFA)/acetonitrile (+0,1% TFA), gradient to 30:70 water (+0,1% TFA)/acetonitrile (+0,1% TFA) in 24 min, to 10:90 water (+0,1% TFA)/acetonitrile (+0,1% TFA) in 10 min, with a 2ml/min flow. The elution of the product was monitored by radiochemical and UV (220 nm) detectors (Figure 18).

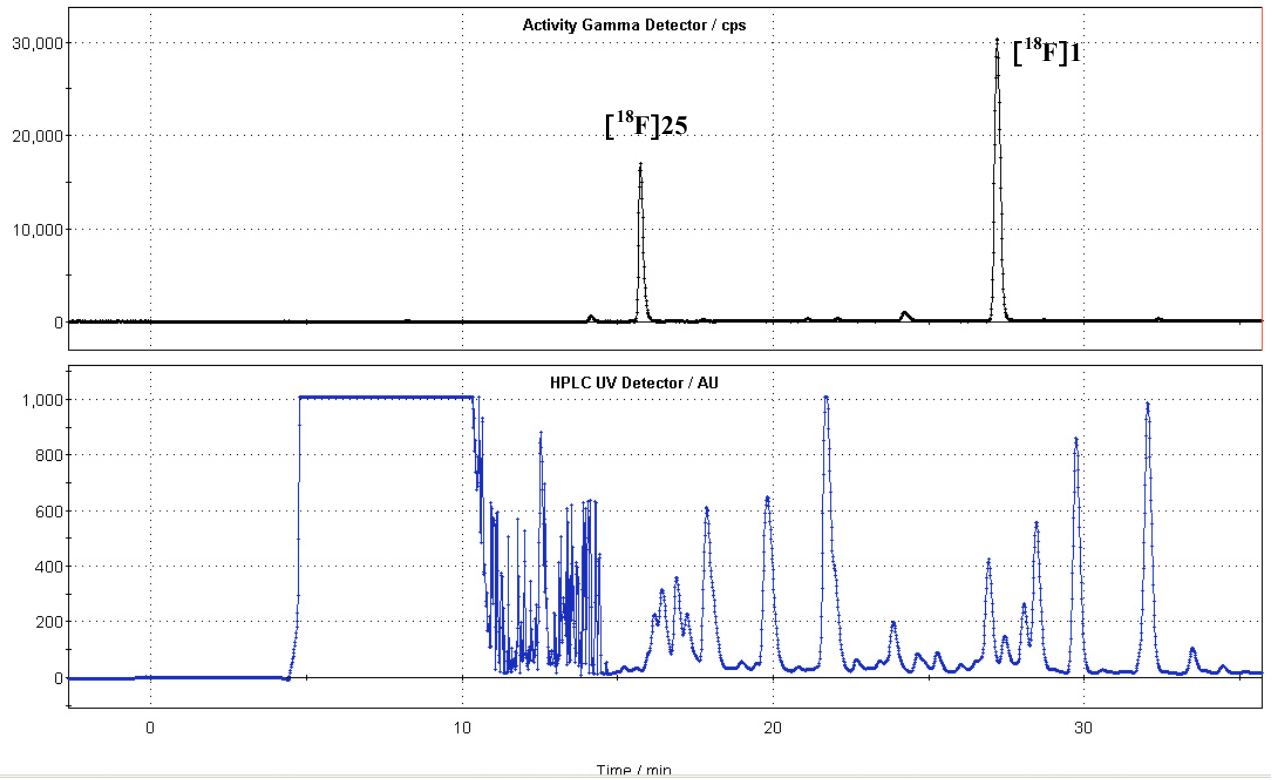


Figure 18

What we could observed was that the conversion to the desired product seemed to be lower considering previously obtained results, but by comparing measured starting radioactivity and HPLC eluted radioactivity, we finally understood that a great amount of product **[^{18}F]25** was not eluted from the column. Also the poor final RCY of the pure collected product was too low as expected from HPLC plot.

In order to get an idea concerning radiochemical and chemical purity of the final desired product, the product fraction (t_R 15.3 min) was analyzed by analytical RP-HPLC (Figure 19).

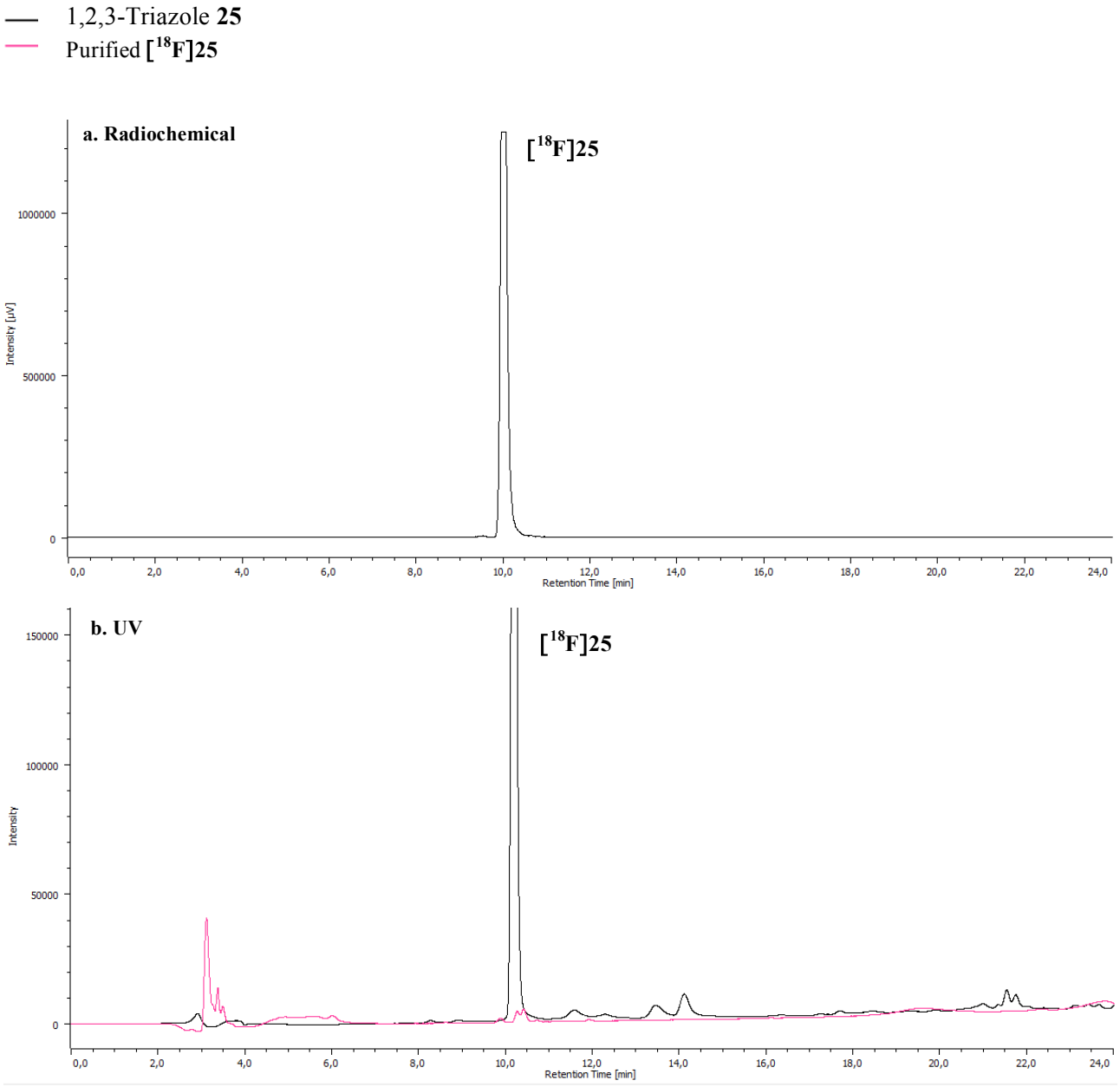


Figure 19

The resulting obtained radiochemical yield for purified product **[^{18}F]25** was only 3%, on the other hand the desired radiolabelled molecule seemed to be extremely pure (100% radiochemical purity, and suitable UV chromatogram). If elution from the column was surely a problem, due to an incomplete solubilization of the reaction mixture, that caused copper aggregates, which clogged the column, on the other hand, the product fraction collection presented problems of mechanic nature. The letters will be solved by opportune modifications of the mechanic system.

It's now clear how problems that we observed during the "cold" reference purification affected also radiolabelled product procedure.

In order to solve purification problems we moved in different directions. A first adjustment we tried was the employment of a quantity ten times lower of iodinate precursor **5** in radiochemical CuAAC, to simplify the subsequent HPLC separation during purification of the final product **[^{18}F]25**, but this method revealed to be not successful, as RCY finally resulted to be too low.

Other methods are currently to be tested and are listed below:

- performing CuAAC in water/ethanol, to simplify final UV chromatogram;
- decreasing of radiolabelling reaction times, to reduce by-products that are collected with **[^{18}F]1** after purification;
- employment of EDTA to sequestrate copper from radiolabelled product, and filtration of the mixture prior HPLC/ Sep-Pak cartridges purification.

CONCLUSIONS

A completely new azido precursor useful for ^{18}F-radiolabelling of interesting biomolecules *via* CuAAC was designed. Compared with analogue precursors described in literature, this compound showed significantly improved characteristics in terms of chemical stability and safety, while maintaining suitable chemical and radiochemical reactivities, and biologic suitability.

The synthesis of such a precursor was successfully carried out. In particular, the synthetic procedure was characterized by a protection-deprotection strategy that finally brought to a suitable azido precursor, that could present three different leaving groups, all potentially useful for ^{18}F-radiolabelling: tosylate, mesylate, and iodinate.

The three kind of leaving group were compared one another by testing precursors in radiolabelling assays, entirely performed using an automated synthesis module. The best precursor was found to be the iodinate. This not only showed the major radiochemical yield (61%, non-corrected for the decay, 85% radiochemical purity) after 20 minutes, but also the best features to be properly purified and used in the following CuAAC.
^{18}F-radiolabelled compound was successfully purified, by a time-effective Sep-Pak cartridges purification method with a 51% radiochemical yield (non-corrected for the decay, 93% radiochemical purity).

The pure ^{18}F-radiolabelled azide was finally tested in CuAAC with an alkynyl-modified biologic model, propargyl glycine. After thirty minutes at room temperature, we observed conversion of 52% into the desired ^{18}F-radiolabelled amino acid, with a 18% overall radiochemical yield (non-corrected for the decay). However, problems dealing with final purification have already to be overtaken, especially concerning copper aggregates and copper complexes of the ^{18}F-radiolabelled click product.

Future developments include the improvement of final ^{18}F-radiolabelled propargylglycine purification, by operating both onto CuAAC conditions and by optimizing copper sequestration from the product complex. The purified ^{18}F-radiolabelled amino acid will be thus tested *in vivo* in

pre-clinic PET assays, in collaboration with Prof. Rosamaria Moresco group of University of Milano-Bicocca, to verify its biodistribution, and pharmacokinetic, as well as its stability *in vivo*.

A *scale-down* of the optimized procedure, in respect of the alkyne amount will be developed, in order to further decrease the final process cost.

The optimized radiolabelled procedure will be thus transferred to biologically interesting molecules such as peptides or oligomers that will be monitored *in vivo* to study biologic and medical aspects. In particular, RGD- or NGR-like peptides will be the most probable substrates on which we will start to operate.

EXPERIMENTAL

MATERIALS AND METHODS

All reagents and solvents were purchased from Sigma-Aldrich.

TLC analysis were performed on silica gel 60 F254 pre-coated plates with a fluorescent indicator (Merck) and on ALUGRAM RP-18W/UV_{254} pre-coated sheets with a fluorescent indicator (Machery-Nagel), with detection by a 5% phosphomolybdic acid solution in ethanol, and heating at 110 °C.

DSCs (Differential Scanning Calorimetry) were registered on a Perkin Elmer instrument (Mod. DSC7), using a heating rate of 10° C/min, from 40° C to 120° C.
Infrared spectra were recorded on a Perkin Elmer instrument (Mod. Spectrum One FT-IR) equipped with the ATR sampling device.

ESI-MS and LC-MS spectra were recorded by a mass spectrometer including an AmaZon ETD (Bruker Daltonics, Bremen, DE) ion trap instrument (ESI positive), using the direct inlet probe technique, or LC technique. The samples were dissolved in methanol or water/acetonitrile at a final concentration of 0.0025 mg/ml and injected at an infusion rate of 0.180 ml/hour, or eluted by an analytical RP-HPLC column (Xterra C18 250x4.6 mm, 5 µm, from Waters Corp.). To perform LC, the device supported two Jasco PU 980 Intelligent HPLC pump modules connected with an LC-net cable. LC apparatus was connected to the ion trap cited above. Parameters for the electrospray source are listed below. Voltage parameters: capillary -4500V, end plate offset -500V, solvation gas: nebulizer 20psi, dry gas 9l/min at 200°C. Ions from the source were analyzed in an interval ranging from 70 to 1000 m/z, with 200000 ICC, and 50ms max accumulation time. A target mass of 300 m/z and a trap drive of 100% were employed. Spectra were acquired using MRM modality and by optimizing both the isolation window and the fragmentation energy for each analyte, and processed by the DataAnalysis™ 4.0 (Bruker Daltonics) software.

NMR spectra were recorded on a Bruker AVANCE 500 spectrometer equipped with a 5mm broadband reverse probe with field *z*-gradient operating at 500.13 and 125.76 MHz for ^{1}H and ^{13}C, respectively. NMR spectra were recorded at 298 K in $CDCl_3$ (isotopic enrichment 99.95%) solution, unless otherwise reported, and the chemical shifts were reported on a δ (ppm) scale. The data were

collected and processed by XWIN-NMR software (Bruker) running on a PC with Microsoft Windows 7. The samples (10 mg), were dissolved in the appropriate solvent (0.7 mL) in a 5 mm NMR tube. Acquisition parameters for 1D were as follows: ^{1}H spectral width of 5000 Hz and 32K data points providing a digital resolution of ca. 0.305 Hz per point, relaxation delay 2 s; ^{13}C spectral width of 29,412 Hz and 64 K data points providing a digital resolution of ca. 0.898 Hz per point, relaxation delay 2 s (unless otherwise indicated). The experimental error in the measured ^{1}H-^{1}H coupling constants was ±0.5 Hz. Chemical shifts (δ) of the ^{1}H NMR and ^{13}C NMR spectra are reported in ppm using the signal for residual solvent protons resonance as internal standard (^{1}H NMR: $CDCl_3$ 7.26, D_2O acidified with DCl (solution 38% in D_2O) 4.79 ppm; ^{13}C NMR: $CDCl_3$ 77.0 (central line), D_2O acidified with DCl (solution 38% in D_2O) 4.79 ppm. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet, and br, broad signal. For two-dimensional experiments, Bruker microprograms using gradient selection (gs) were applied. All two-dimensional spectra (COSY, HSQC) were acquired with 2048 data points for t_2 and 256 for t_1 increments.

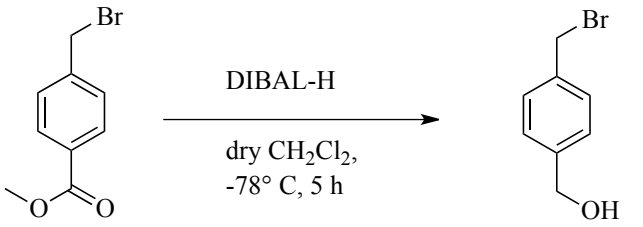
[^{18}F] Fluoride was produced by a cyclotron 18/9 (IBA Cyclone 18/9) via the $^{18}O(p,n)^{18}F$ nuclear reaction, by irradiation of 2 mL water target containing >87% enriched [^{18}O]water (Rotem). Radioactive tests were carried out on a commercially available radiochemistry automated module (GE TracerLab FX N Pro).

Sep-Pak Light Waters Accel Plus QMA, Sep-Pak alumina N light, and SepPak tC18 cartridges were from Waters Corp.

Radiolabeled preparations and unlabeled references were analyzed by RP-HPLC on a Jasco PU-2089i system equipped with an automated injector, DAD detector, and radiochemical detector Raytest Gabi Star. Semipreparative purification was carried out on a RP-HPLC Perkin Elmer Flexar system connected to the automated module for radiosynthesis, and equipped with Knauer WellChrom Spectro-Photometer K-2501. Absorbances for all compounds were detected at 220 nm. Analytical RP-HPLC column (Xterra C18 250x4.6 mm, 5 μm), and semipreparative RP-HPLC column (XBridge PREP C18, 150x10 mm, 5 μm) were bought from Waters Corp.

PREPARATION OF UNLABELLED INTERMEDIATES

[4-(Bromomethyl)phenyl]methanol (11)



11 y 92%

The synthesis was carried out similarly as described in literature. [58] A solution of diisobuthyl aluminium hydride 1M in tetrahydrofuran (50 ml) was added dropwise under nitrogen into a cooled-down solution (-78 °C) of methyl 4-(bromomethyl)benzoate (3.48 g, 15.2 mmol) in anhydrous dichloromethane (90 ml). The mixture was stirred at -78 °C for 4 h, and then diisobuthyl aluminium hydride solution (5 ml) was added. The reaction progress was monitored by TLC (toluene/ethyl acetate 9:1). After 1 h the reaction went to completion. The mixture was kept at 0 °C while quenching with water, then it was allowed to warm to room temperature. The organic layer was separated, and the aqueous one was extracted with dichloromethane (2 x 50 ml). The combined organic extract were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to afford the desired product **11** (2.82 g, 14.0 mmol, 92% yield) as a white solid.

^{1}H-NMR (500MHz, $CDCl_3$) δ 1.87 (br s, 1H, exchangeable with D_2O), 4.50 (s, 2H, ArC*H*$_2$Br), 4.68 (s, 2H, ArC*H*$_2$O), 7.33 (d, 2H, Ar), 7.39 (d, 2H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 33.23 (Ar*C*H$_2$Br), 64.82 (Ar*C*H$_2$O), 127.28, 129.22, 137.13, 141.14

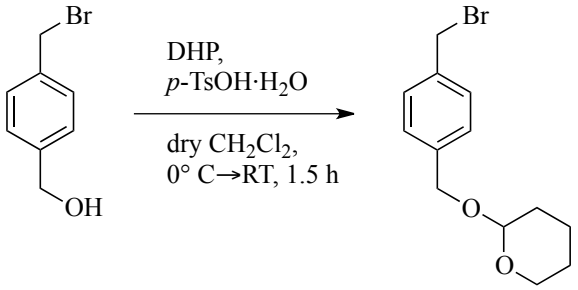
These product **11** NMR data are in agreement with the reported ones. [87]

R_f 0.28

IR: ν 3320, 1419, 1224, 1194, 1006, 831 cm^{-1}

DSC: endothermic peak at 86.8° C

2-{[4-(Bromomethyl)benzyl]oxy}tetrahydro-2*H*-pyran (13)



11 **13** y 94%

The synthesis was carried out similarly as described in literature. [61] A solution of compound **11** (2.0 g, 9.95 mmol, 1 eq) and 3,4-dihydro-2*H*-pyran (2.4 ml, 25.9 mmol, 2.6 eq) in anhydrous dichloromethane (50 ml) was cooled-down to 0 °C with an ice bath. After the addition of monohydrate *p*-toluensulfonic acid (0.010 g, 0.05 mmol, 0.005 eq), the reaction mixture was stirred 10 min at 0 °C, and then at room temperature for 1.5 h. The completion of the reaction was monitored by TLC (petroleum ether/ethyl acetate 9:1). The organic layer was washed with a mixture of saturated brine/saturated sodium hydrogen carbonate/water 1:1:2 (50 ml), and with saturated brine (10 ml x 2). The organic layer was then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The obtained yellow oil (3.2 g) was purified by column chromatography on silica gel (silica/crude product 8:1, gradient elution from petroleum ether 100% to petroleum ether/ethyl acetate 95:5), to afford the desired product **13** (2.7 g, 9.35 mmol, 94% yield) as yellow oil.

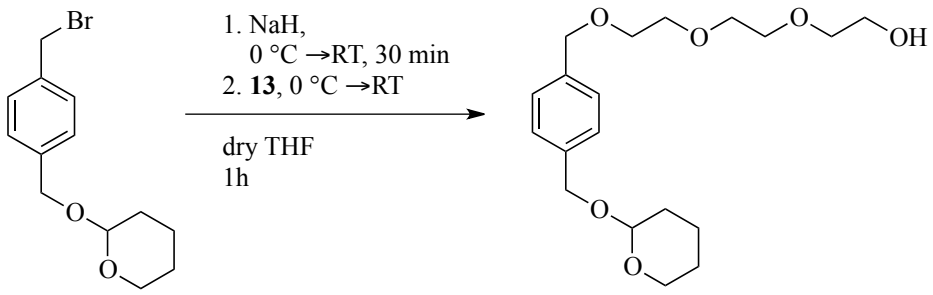
R_f 0.59

^{1}H-NMR (500MHz, $CDCl_3$) δ 1.44-1.95 (m, 6H, 3CH_2), 3.49-3.61 (m, 1H, CH_aOCHO), 3.87-3.95 (m, 1H, CH_bOCHO),), 4.50 (s, 2H, ArCH_2Br), 4.50 (d, 1H, ArCH_aOTHP), 4.71 (t, 1H, OCHO), 4.78 (d, 1H, ArCH_bOTHP) 7.34 (d, 2H Ar) 7.38 (d, 2H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 19.29, 25.43, 30.52, 33.35 (Ar*C*H_2Br), 62.11 (*C*H_2OCHO), 68.33 (Ar*C*H_2OTHP), 97.80 (OCHO), 128.11, 129.07, 136.95, 138.73

These product **13** NMR data are in agreement with the reported ones. [88]

2-{2-[2-({4-[(tetrahydro-2*H*-pyran-2-yloxy)methyl]benzyl}oxy)ethoxy]ethoxy}ethanol (16)



13 **16** y 43%

A solution of triethylene glycol (1.29 g, 8.6 mmol, 1 eq) in anhydrous tetrahydrofuran (70 ml), under nitrogen, was cooled-down to 0 °C. Sodium hydride (0.21 g, 8.6 mmol) was added, and the reaction mixture was stirred for 10 min at 0 °C, then it was allowed to warm to room temperature, and it was finally stirred for 30 min. After cooling-down to 0 °C, a solution of compound **13** (2.46 g, 8.6 mmol, 1 eq) in tetrahydrofuran (30 ml) was added dropwise, and the reaction mixture was then stirred at room temperature overnight. The reaction progress was monitored by TLC (toluene/ethyl acetate 9:1 to verify the disappearance of compound **13**, and petroleum ether/ethanol 8:2 to monitor the presence of the product). Sodium hydride was quenched with water cooling the reaction mixture in an ice bath; the mixture was concentrated under reduced pressure. After dilution with dichloromethane, the organic layer was washed twice with water, dried over anhydrous sodium sulfate, and filtered. The solvent was finally evaporated under reduced pressure, to obtain the crude product (3,0 g), that was then purified by column chromatography on aluminum oxide (4.7% water content)(10:1), gradient elution from petroleum ether/ethyl acetate 9:1 to 100% ethyl acetate, to afford the desired product **16** (1.3 g, 3.7 mmol, 43% yield).

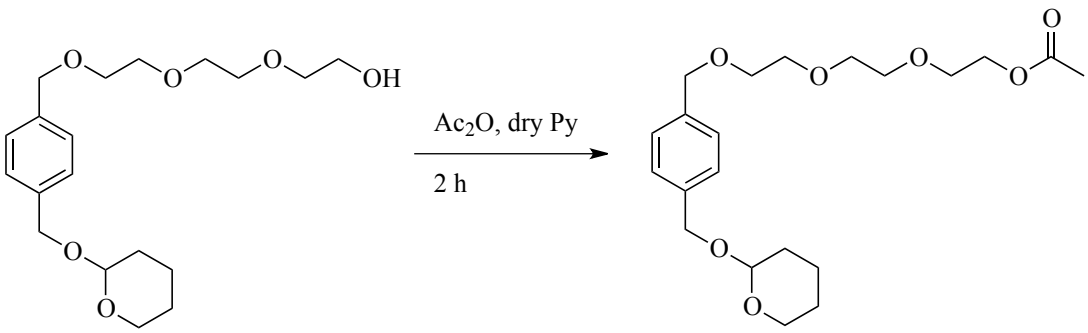
R_f 0.61

^{1}H-NMR (500MHz, $CDCl_3$) δ 1.44-1.93 (m, 6H, 3CH_2), 2.64 (br s, 1H, exchangeable with D_2O), 3.71-3.77 (m, 12H, CH_2O), 3.72 (dt, 2H, CH_2OCHO), 4.48 (d, 1H, ArCH_aOTHP), 4.55 (s, 2H, ArCH_2O), 4.76 (d, 1H, ArCH_bOTHP), 4.69 (m, 1H, OCHO), 7.28-7.37 (m, 4H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 19.29, 25.42, 30.51, 61.69 (CH_2OH), 62.06 (*C*H_2OCHO), 68.49 (Ar*C*H_2OTHP), 69.29, 70.34, 70.57, 70.62, 72.46, 72.98 (Ar*C*H_2O), 97.62 (OCHO), 127.76, 127.82, 137.39, 137.68

IR: ν 3449, 2867, 1016, 811 cm^{-1}

2-{2-[2-({4-[(tetrahydro-2*H*-pyran-2-yloxy)methyl]benzyl}oxy)ethoxy]ethoxy}ethyl acetate (18)



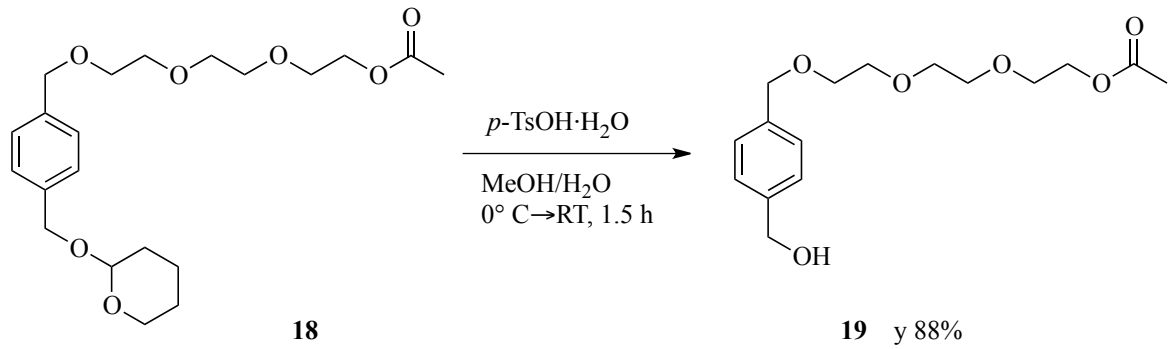
16 **18** y 99%

To a solution of **16** (1.3 g, 3.7 mmol, 1 eq) in anhydrous pyridine (5 ml) acetic anhydride was added (1.2 g, 11.8 mmol, 3.2 eq). The reaction mixture was stirred at room temperature for 2 h, and monitored by TLC (petroleum ether/ethanol 8:2). After dilution with water (water/pyridine 5:1), extraction with dichloromethane was carried out (25 ml x 3). The organic layer was dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated under reduced pressure. The obtained crude product **18** (1.45 g, 3.66 mmol , 99% yield) was employed in the following step without further purifications.

R_f 0.82

^{1}H-NMR (500MHz, $CDCl_3$) δ 1.46-1.92 (m, 6H, 3CH_2), 2.07 (s, 3H, CH_3CO), 3.55 (m, 1H, CH_aOCHO), 3.59-3.74 (m, 10H, CH_2O), 3.85-3.98 (m, 1H, CH_bOCHO), 4.22 (t, 2H, CH_2OCO), 4.56 (s, 2H, ArCH_2O), 4.50 (d, 1H, ArCH_aOTHP), 4.78 (d, 2H, ArCH_bOTHP), 4.70 (t, 1H, OCHO), 7.32 (d, 2H, Ar), 7.34 (d, 2H, Ar)

2-[2-(2-{[4-(hydroxymethyl)benzyl]oxy}ethoxy)ethoxy]ethyl acetate (19)



18 **19** y 88%

A solution of compound **18** (0.9 g, 2.3 mmol, 1 eq) in methanol (10 ml) was cooled-down to 0° C. A solution of monohydrate *p*-toluensulfonic acid (0.11 g, 0.55 mmol, 0.24 eq) in methanol (10 ml) and water (1 ml) was added dropwise. After 10 min, the mixture was allowed to warm to room temperature, and it was stirred for 1.5 h. The removal of the protecting group was monitored by TLC (petroleum ether/ethanol 8:2). The reaction mixture pH was adjusted to 7 with saturated potassium hydrogen carbonate, then the solvent was evaporated under reduced pressure. Dichloromethane (20 ml) was added, then the organic layer was washed twice with water (10 ml), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure, to afford the crude product (0.69 g), which was finally purified on column on silica gel chromatography (silica/crude product 10:1, gradient elution from petroleum ether/ethyl acetate 9:1 to 100% ethyl acetate), to afford the desired product **19** (3.12 g, 2.02 mmol, 88% yield) as an oil.

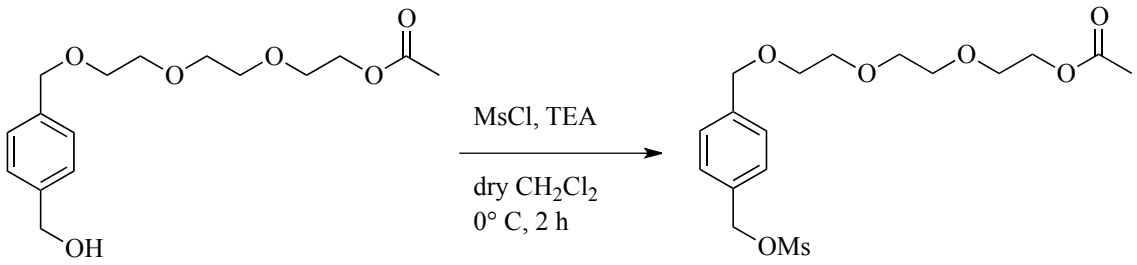
R_f 0.25

^{1}H-NMR (500MHz, $CDCl_3$) δ 1.85 (t, 1H, exchangeable with D_2O), 2.06 (s, 2H, CH_3CO), 3.59-3.72 (m, 10H, CH_2O), 4.19 (t, 2H, CH_2OCO), 4.56 (s, 2H, $ArCH_2O$), 4.67 (d, 2H, $ArCH_2OH$), 7.33 (br s, 4H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 20.91 (CH_3), 63.61 (CH_2OCO), 65.09 (CH_2OH), 69.14, 69.42, 70.57, 70.66, 70.68, 72.96 ($ArCH_2O$), 127.02, 127.95, 137.69, 140.35, 171.07 (C=O)

IR: ν 3452, 2867, 1735, 1232, 1092, 1048 cm^{-1}

2-(2-{2-[(4-{[(methylsulfonyl)oxy]methyl}benzyl)oxy]ethoxy}ethoxy)ethyl acetate (20)



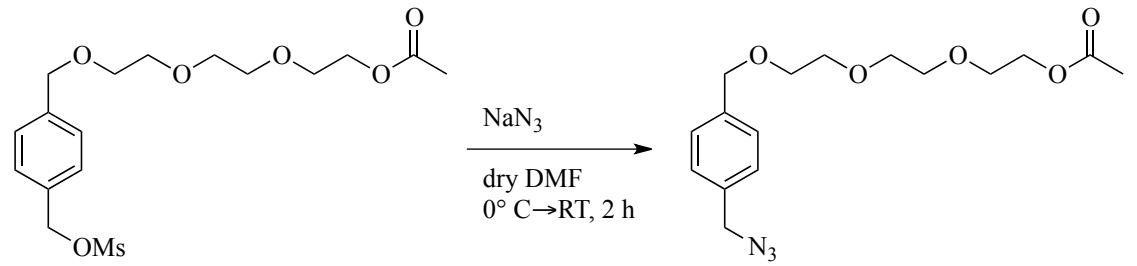
19 **20** y 93%

To a solution of compound **19** (0.88 g, 2.8 mmol, 1 eq) in anhydrous dichloromethane (20 ml), under nitrogen, triethylamine (585 µl, 4.2 mmol, 1.5 eq) was added, and the temperature was cooled down to 0° C. Methanesulfonylchloride (0.39 g, 3.4 mmol, 1.2 eq) was slowly added, and the reaction mixture was stirred at 0° C for 2 h. The reaction progress was monitored by TLC (petroleum ether/ethanol 8:2). The reaction mixture was diluted with dichloromethane (20 ml), and the organic layer was washed twice with saturated brine (40 ml), once with water (40 ml), dried over anhydrous sodium sulfate, and filtered. The solvent was evaporated under reduced pressure, to afford the crude product **20** (1.02 g, 2.60 mmol, 93% yield) as an oil.

R_f 0.13

^{1}H-NMR (500MHz, $CDCl_3$) δ 2.07 (s, 3H, CH_3CO), 2.92 (s, 3H, CH_3SO_2), 3.60-3.74 (m, 10H, CH_2O), 4.22 (t, 2H, CH_2OCO), 4.59 (s, 2H, ArCH_2O), 5.24 (s, 2H, CH_2OSO_2), 7.39 (br s, 4H, Ar)

2-[2-(2-{[4-(azidomethyl)benzyl]oxy}ethoxy)ethoxy]ethyl acetate (21)



20 **21** y 97%

A solution of compound **20** (1.02 g, 2.61 mmol, 1 eq) in anhydrous dimethylformamide (3 ml), kept under nitrogen, was cooled-down to 0° C. Sodium azide (0.18 g, 2.74 mmol, 1.05 eq) is then added,

and the reaction mixture was stirred at room temperature for 2h. The reaction progress was monitored by TLC (petroleum ether/ethanol 8:2). The reaction mixture was diluted with water (30 ml) and extracted with ethyl acetate (3 x 15 ml). The collected organic phases were washed with saturated brine (2 x 15 ml), then dried over anhydrous sodium sulfate, and filtered. The solvent was evaporated under reduced pressure to afford the crude product **21** (0.85 g, 2.53 mmol, 97% yield) as an oil.

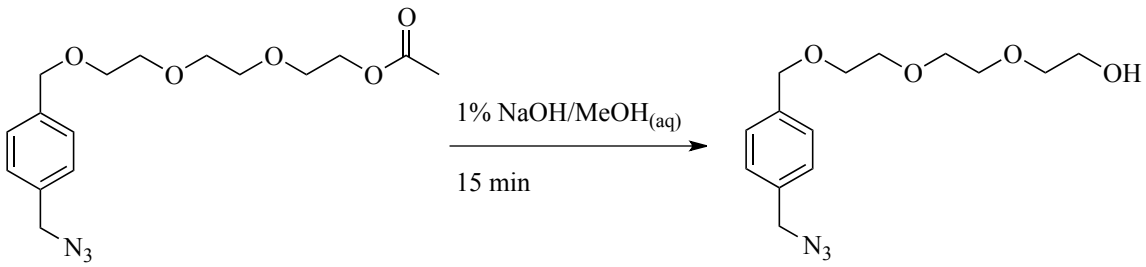
R_f 0.79

^{1}H-NMR (500MHz, $CDCl_3$) δ 2.07 (s, 3H, CH_3CO), 3.60-3.73 (m, 10H, CH_2O), 4.22 (t, 2H, CH_2OCO), 4.32 (s, 2H, CH_2N_3), 4.57 (s, 2H, ArCH_2O), 7.29 (d, 2H, Ar), 7.36 (d, 2H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 20.93 (CH_3), 54.54 (ArCH_2O), 63.59 (CH_2OCO), 69.14, 69.57, 70.59, 70.64, 70.68, 72.81 (CH_2N_3), 128.10, 128.27, 134.66, 138.51, 171.02 (C=O)

IR: ν 2868, 2095, 1233, 1096, 1051 cm^{-1}

2-[2-(2-{[4-(azidomethyl)benzyl]oxy}ethoxy)ethoxy]ethanol (**22**)



21 **22** y 86%

Compound **21** (0.80 g, 2.4 mmol, 1 eq) was suspended in a solution of sodium hydroxide (0.12 g) in 15 ml aqueous methanol, and stirred for 15 min at room temperature. The reaction progress was monitored by TLC (petroleum ether/ethanol 8:2). The pH was adjusted to 7-8 with a solution of hydrochloric acid 1 M. The solvent was evaporated, and dichloromethane (50 ml) was added. The organic layer was washed with water (3 x 8 ml), then dried over anhydrous sodium sulfate, and filtered. The solvent was evaporated under reduced pressure, to obtain the crude product (0.67 g), which was finally purified by column chromatography on silica gel (silica/crude product 10:1, gradient elution from petroleum ether/ethyl acetate 9:1 to 100% ethyl acetate), to afford the desired product **22** (0.61 g, 2.06 mmol, 86% yield) as a colorless oil.

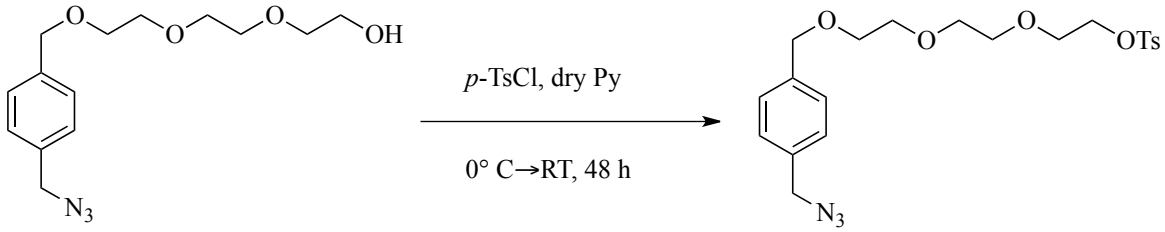
R_f 0.49

^{1}H-NMR (500MHz, $CDCl_3$) δ 2.22 (br s, 1H, exchangeable with D_2O), 3.57-3.78 (m, 12H, CH_2O), 4.32 (s, 2H, CH_2N_3), 4.57 (s, 2H, ArCH_2O), 7.29 (d, 2H, Ar), 7.37 (d, 2H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 54.52 (CH_2N_3), 61.74, 69.49, 70.34, 70.57, 70.65, 72.48 (CH_2OH), 72.83 (ArCH_2O), 128.15, 128.28, 134.69, 138.37

IR: ν 3454, 2867, 2094, 1248, 1090, 1065, 806 cm^{-1}

2-[2-(2-{[4-(azidomethyl)benzyl]oxy}ethoxy)ethoxy]ethyl 4-methylbenzenesulfonate (**2**)



22 **2** y 16%

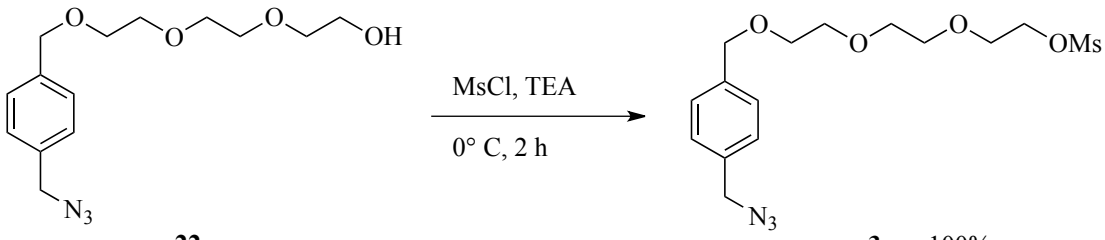
A solution of compound **22** (0.043 g, 0.15 mmol, 1 eq) in anhydrous pyridine (2 ml) was cooled-down to 0° C. *p*-Toluensulfonyl chloride (0.044 g, 0.23 mmol, 1.5 eq) was added and after 24h at room temperature, under stirring, an additional amount of p-TsCl (0.044 g) was added, and the reaction mixture was kept under stirring at room temperature for 24 h. The reaction progress was monitored by TLC (petroleum ether/ethanol 8:2). The reaction mixture was diluted with water/ice (10 ml), and extracted with dichloromethane (3 x 10 ml). The collected organic layers were washed with water (3 x 10 ml), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure, to afford the crude product (0.042 g), that was finally purified by column chromatography on silica gel (silica/crude product 20:1, gradient elution from petroleum ether/ethyl acetate 95:5 to 6:4), to afford the desired product **2** (0.011 g, 0.024 mmol, 16% yield) as a colorless oil.

R_f 0.54

^{1}H-NMR (500MHz, $CDCl_3$) δ 2.43 (s, 3H, CH_3Ar), 3.56-3.74 (m, 10H, CH_2O), 4.15 (t, 2H, CH_2OTs), 4.33 (s, 2H, CH_2N_3), 4.56 (s, 2H, ArCH_2O), 7.27-7.41 (m, 6H, Ar), 7.79 (d, 2H, Ar)

LC-MS: m/z 472, 523

2-[2-(2-{[4-(azidomethyl)benzyl]oxy}ethoxy)ethoxy]ethyl methanesulfonate (3)



22 **3** y 100%

Compound **22** (0.41 g, 1.39 mmol, 1 eq) was suspended in anhydrous dichloromethane (10 ml), under nitrogen. Triethylamine (0.3 ml, 2.09 mmol, 1.5 eq) was added, and the solution was cooled-down to 0° C. Mesyl chloride (0.19 g, 1.67 mmol, 1.2 eq) was added dropwise, and the reaction mixture was stirred for 2 h at 0° C. The reaction progress was monitored by TLC (petroleum ether/ethanol 8:2). The reaction mixture was diluted with dichloromethane (10 ml), and the organic layer was washed once with saturated brine (20 ml), and once with water (20 ml), then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure, to afford the desired product **3** (0.52 g, 1.39 mmol, 100% yield) as a colorless oil.

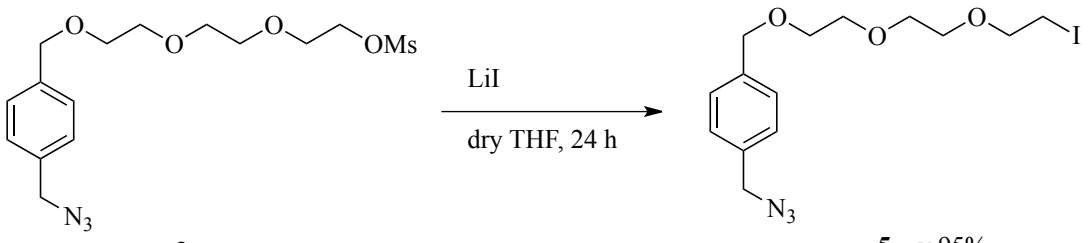
R_f 0.40

^{1}H-NMR (500MHz, $CDCl_3$) δ 3.04 (s, 3H, CH_3SO_2), 3.58-3.72 (m, 8H, CH_2O), 3.77 (br s, 2H, CH_2O), 4.33 (s, 2H, CH_2N_3), 4.37 (br s, 2H, C*H*$_2$OMs), 4.57 (s, 2H, ArC*H*$_2$O), 7.30 (d, 2H, Ar), 7.36 (d, 2H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 37.70 (CH_3SO_2), 54.53 (CH_2N_3), 69.04, 69.23, 69.57, 70.60, 70.64, 70.67, 72.84 (Ar*C*H$_2$O), 128.14, 128.31, 134.75, 138.42

IR: ν 2919, 2095, 1348, 1249, 1172, 1095, cm^{-1}

1-(azidomethyl)-4-({2-[2-(2-iodoethoxy)ethoxy]ethoxy}methyl)benzene (5)



3 **5** y 95%

To a solution of compound **3** (0.16 g, 0.43 mmol, 1 eq) in anhydrous tetrahydrofuran (5 ml) under nitrogen lithium iodide (0.29 g, 2.15 mmol, 5 eq) was added. The reaction mixture was stirred at room temperature overnight and controlled by TLC (petroleum ether/ethanol 8:2). Water was added to quenched the reaction, and tetrahydrofuran was evaporated. Dichloromethane (10 ml) was added, the organic layer was washed with water (3 x 10 ml), then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure, to afford the desired product **5** (0.17 g, 0.41 mmol, 95% yield) as a yellow oil.

R_f 0.8

^{1}H-NMR (500MHz, $CDCl_3$) δ 3.26 (t, 2H, CH_2I), 3.62-3.73 (m, 8H), 3.76 (t, 2H, OCH_2CH_2I), 4.33 (s, 2H, CH_2N_3), 4.58 (s, 2H, $ArCH_2O$), 7.30 (d, 2H, Ar), 7.37 (d, 2H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 2.87 (CH_2I), 54.57 (CH_2N_3), 69.62, 70.27, 70.68, 70.74, 72.02 (OCH_2CH_2I), 72.85 ($ArCH_2O$), 128.12, 128.26, 134.68, 138.55

IR: ν 2863, 2094, 1251, 1090, 805, 672 cm^{-1}

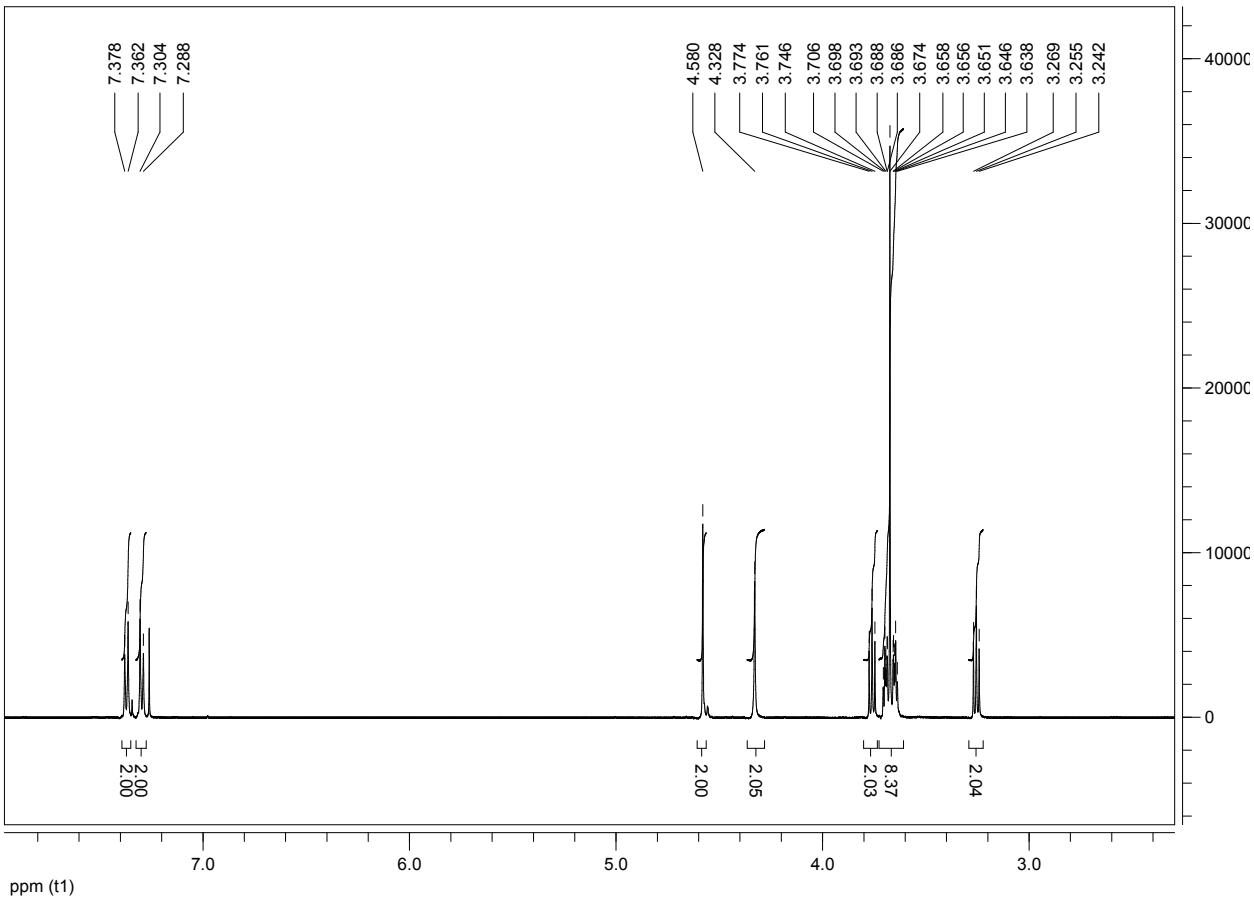


Figure 20 – ^{1}H-NMR compound 5

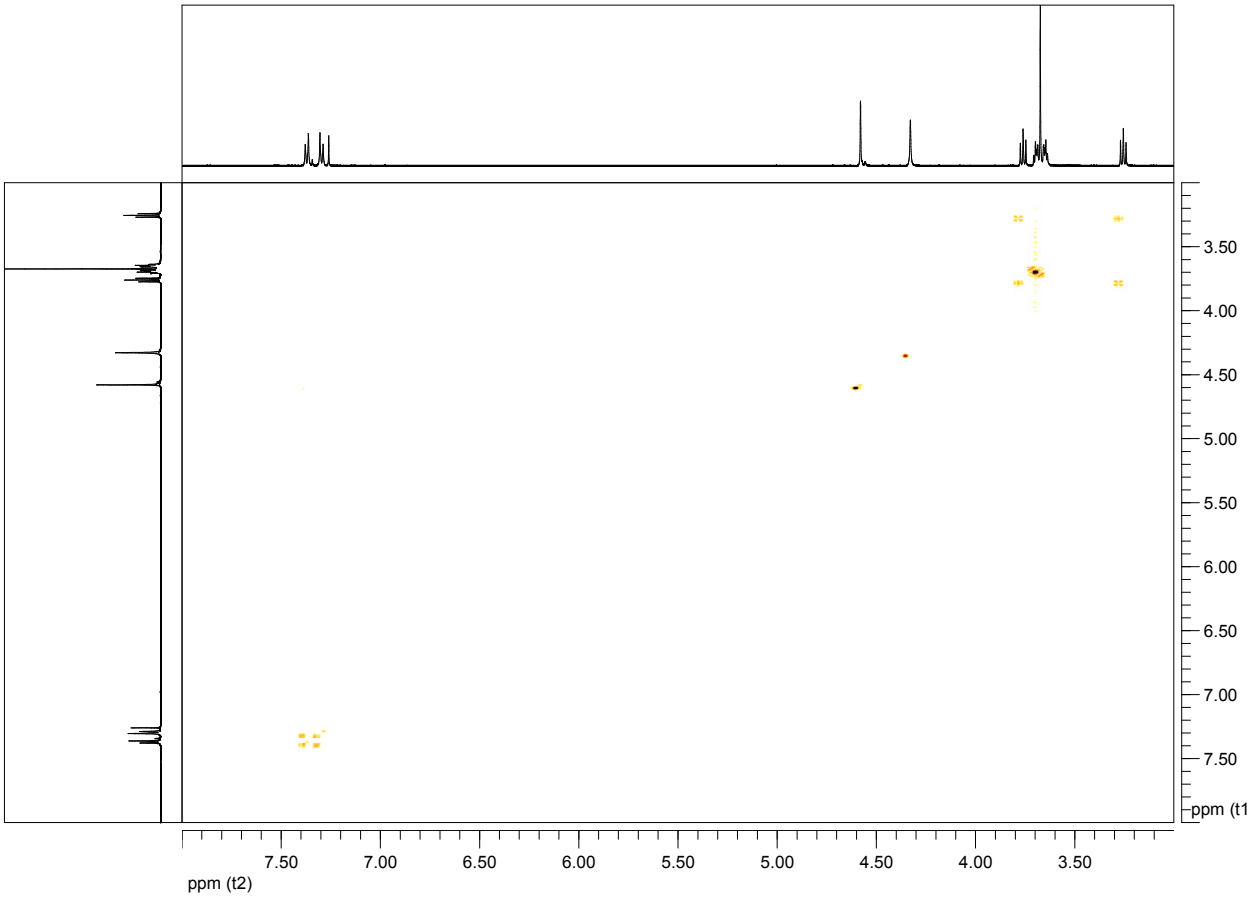


Figure 21 – COSY-NMR compound 5

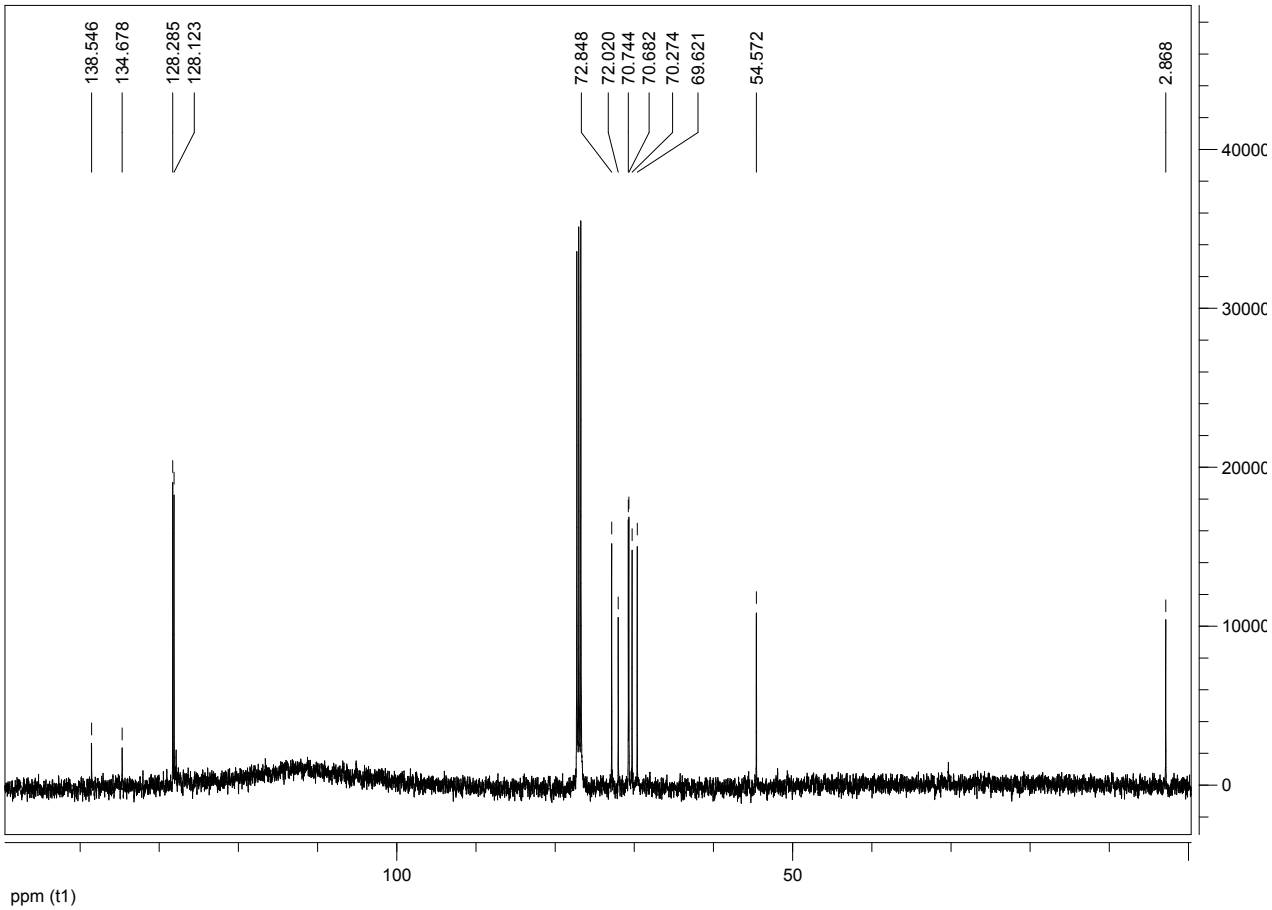


Figure 22 – ^{13}C-NMR compound 5

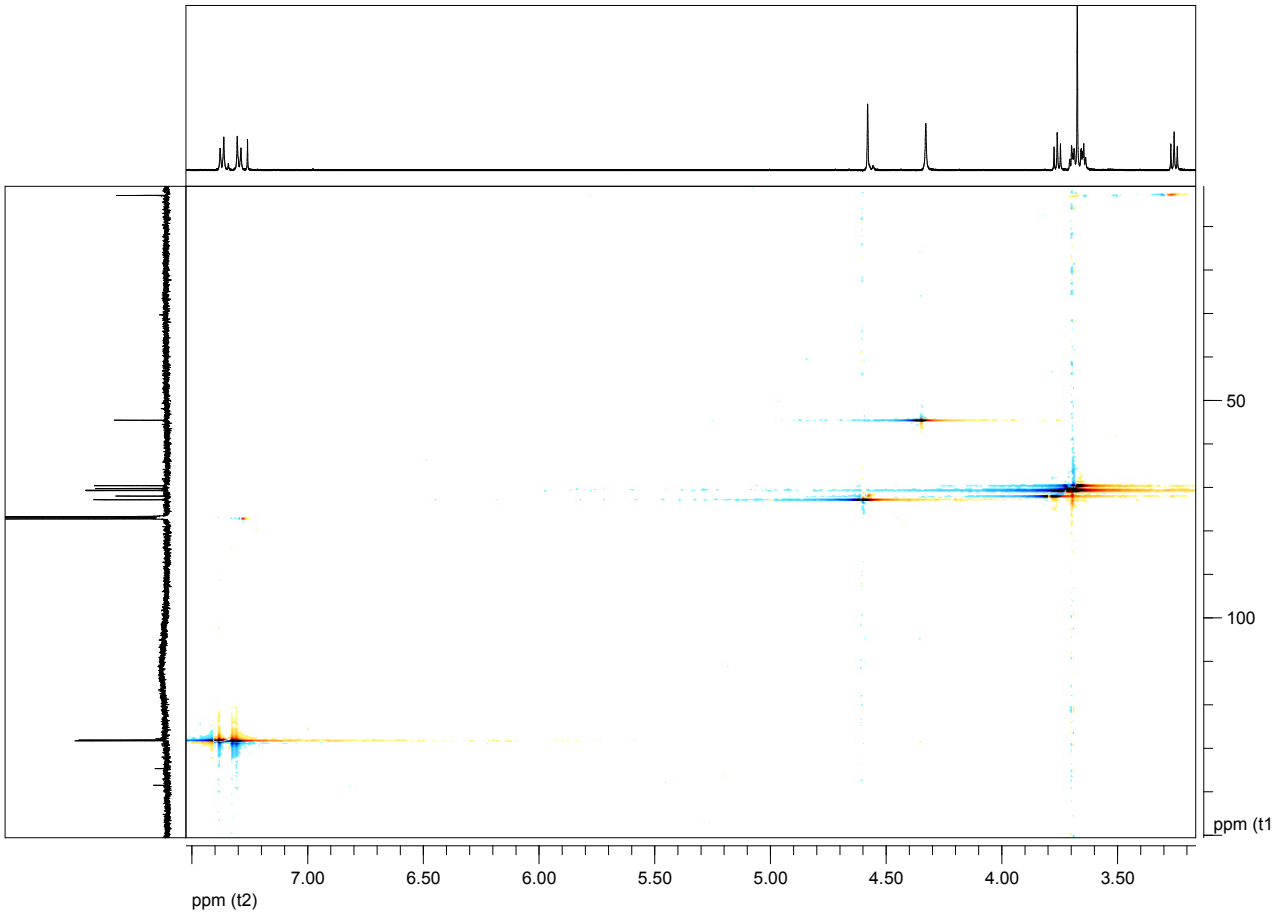
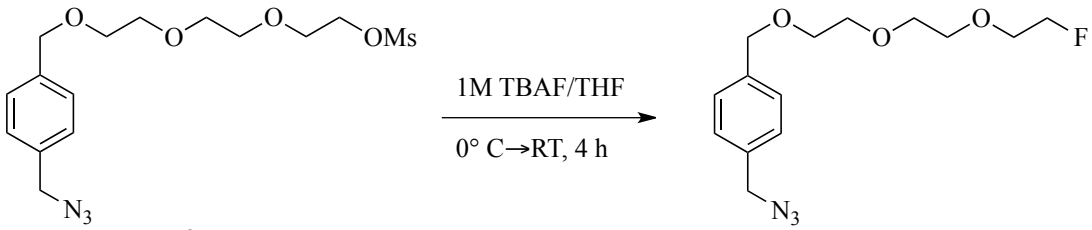


Figure 23 – HSQC-NMR compound 5

1-(azidomethyl)-4-({2-[2-(2-fluoroethoxy)ethoxy]ethoxy}methyl)benzene (1)



3 **1** y 71%

Compound **3** (0.18 g, 0.48 mmol, 1 eq) was dissolved in anhydrous tetrahydrofuran (2 ml), and the solution was cooled-down to 0° C under nitrogen stream. A solution of tetrabutylammonium fluoride 1 M in tetrahydrofuran (0.1 ml, 0.96 mmol, 2 eq) was added, and after the reaction mixture was allowed to warm to room temperature, it was stirred for 4 h. The reaction progress was monitored by TLC (petroleum ether/ethanol 8:2). The solvent was evaporated, and dichloromethane (10 ml) was added. The organic layer was washed twice with water (10 ml), once with saturated

brine (10 ml), then dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure, to afford the crude product (0.19 g). Column chromatography on silica gel (silica/crude product 20:1, gradient elution from petroleum ether/ethyl acetate 9:1 to 7:3) was performed to afford the desired product **1** (0.10 g, 0.34 mmol, 71% yield) as colorless oil.

R_f 0.60

^{1}H-NMR (500MHz, $CDCl_3$) δ 3.62-3.71 (m, 8H, CH_2O), 3.75 (dt, 2H, C*H*$_2$CH$_2$F), 4.33 (s, 2H, ArC*H*$_2$O), 4.56 (dt, 2H, CH_2F), 4.58 (s, 2H, CH_2N_3), 7.29 (d, 2H, Ar), 7.37 (d, 2H, Ar)

^{13}C (500MHz, $CDCl_3$) δ 54.57 (Ar*C*H$_2$O), 69.60, 70.34, 70.50, 70.70, 70.86, 72.82 (CH_2N_3), 82.47 and 83.81 (CH_2F), 128.11, 128.27, 134.67, 138.57

IR: ν 2952, 2095, 1248, 1096, 1046, 806 cm^{-1}

TSI-MS: m/z 186, 320, 524

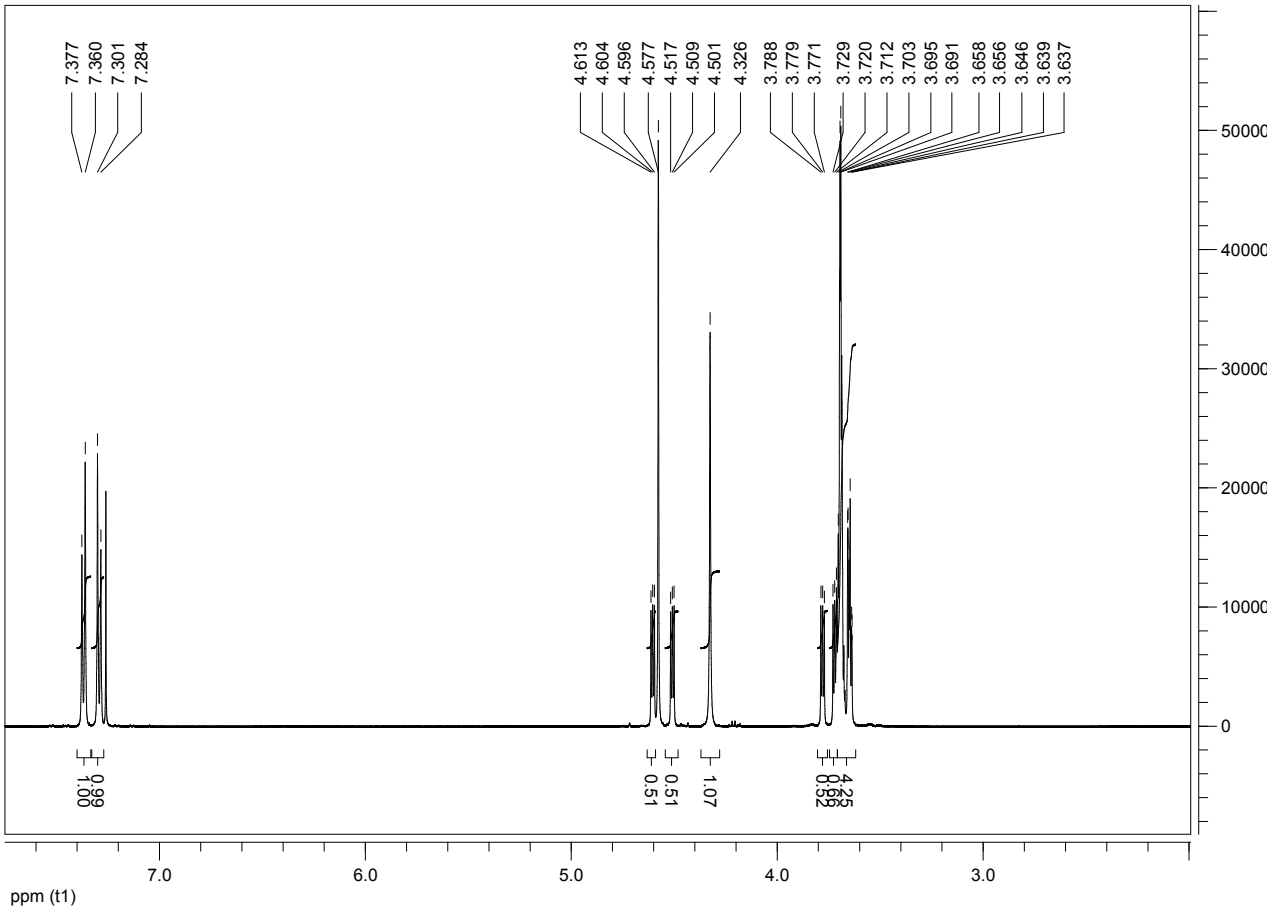


Figure 24 – ^{1}H-NMR compound 1

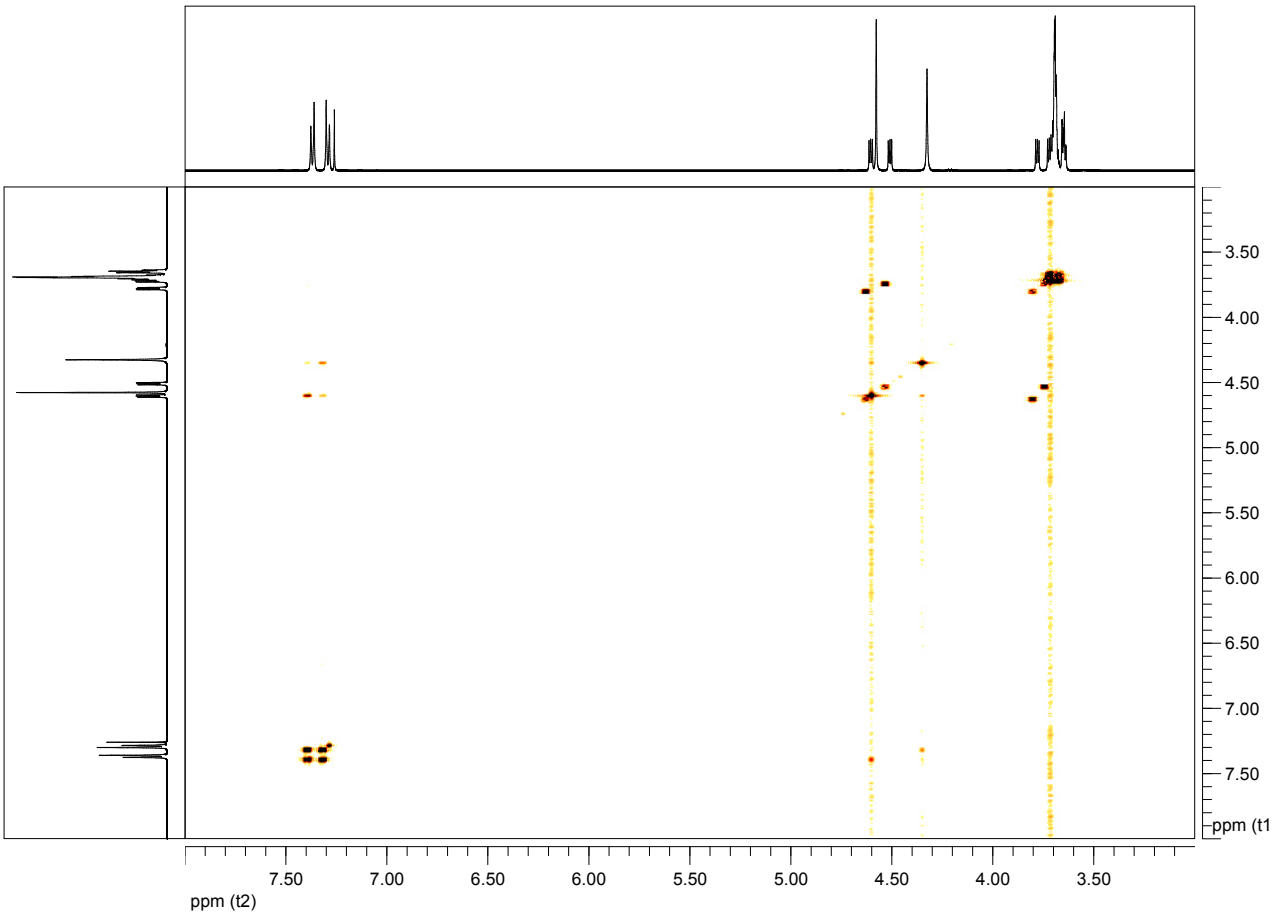


Figure 25 – COSY-NMR compound 1

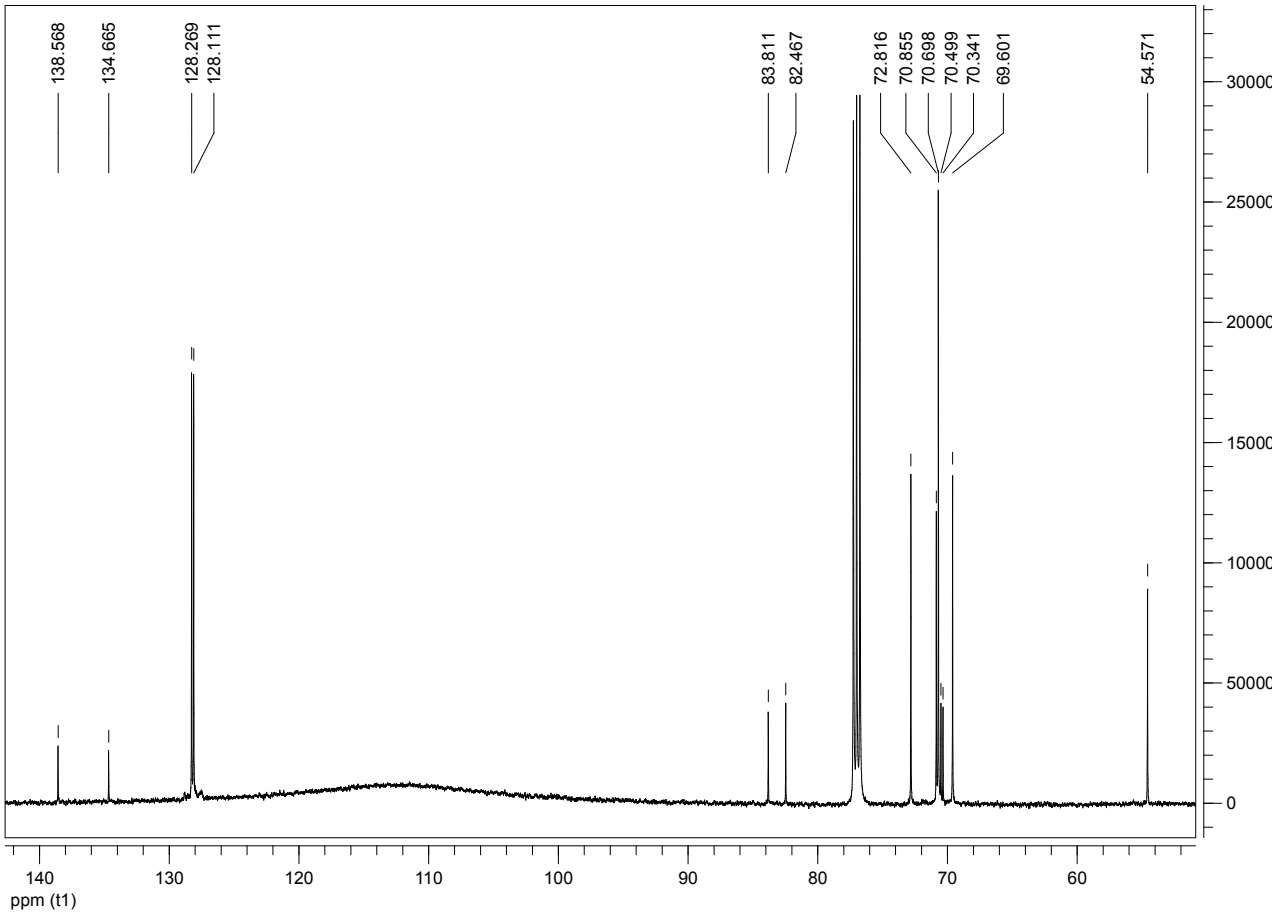


Figure 26 – ^{13}C-NMR compound 1

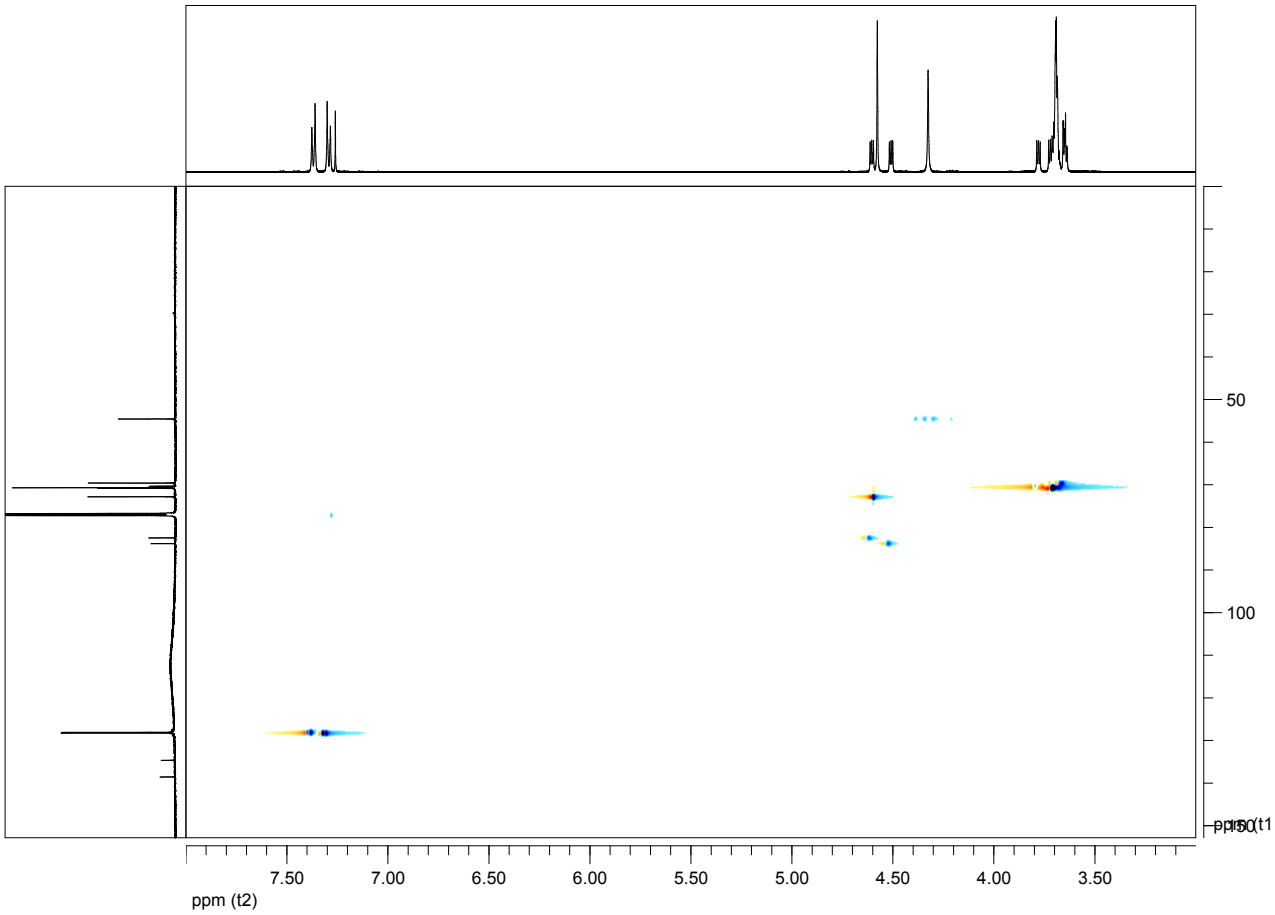
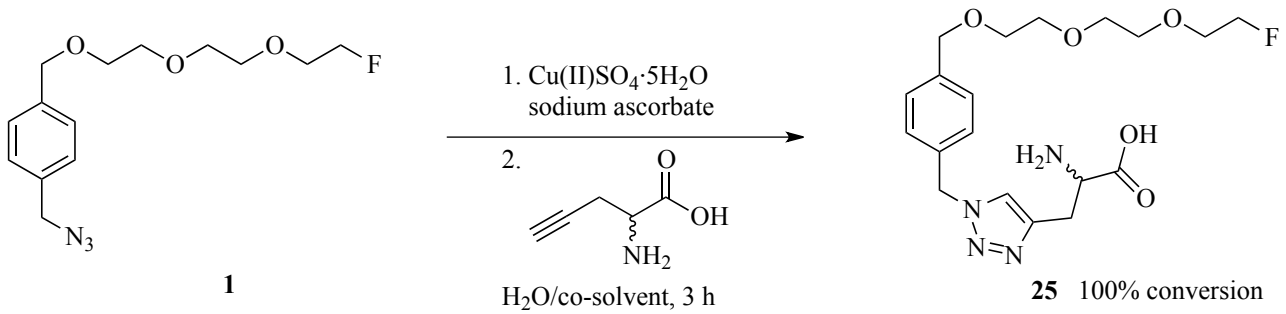


Figure 27 – HSQC-NMR compound 1

3-{1-[4-({2-[2-(2-fluoroethoxy)ethoxy]ethoxy}methyl)benzyl]-1*H*-1,2,3-triazol-4-yl}alanine (25)



A fresh-prepared solution of copper(II) sulfate pentahydrate (10.7 mg, 0.043 mmol, 1.8 eq) in 150 µl water and a fresh-prepared solution of sodium ascorbate (25.7 mg, 0.130 mmol, 5.4 eq) in 150 µl water were mixed together under Helium. After 2 min a solution of propargylglycine (3.3 mg, 0.029 mmol, 1.2 eq) in 450 µl water was added. After 2 min a solution of compound **1** (7.1 mg, 0.024 mmol, 1 eq) in 200 µl acetonitrile and 100 µl dimethylsulfoxide was added. The reaction mixture

was stirred at room temperature for 3 h. The completion of the reaction was monitored by RP-TLC (water/acetonitrile 9:1), and by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 90:10 to 30:70 in 24 min, 1 ml/min, 220 nm). The reaction mixture was solubilized by adding some drops of HCl 1M, and suspended in 20 ml water/acetonitrile 95:5. The solution was passed through a Sep-Pak tC18 plus cartridge, which was then washed with water (10 ml), and with a solution of water/acetonitrile 95:5 (10 ml). The desired compound **25** (6.1 mg, 0.015 mmol, 62% yield) was obtained from elution with pure acetonitrile or ethanol (3 ml), and final evaporation of the solvent.

R_f 0.41

t_R 8-11 min

^{1}H-NMR (500MHz, D_2O+DCl) δ 3.37 (d, 2H, C*H*$_2$Triazole), 3.55-3.66 (m, 8H, CH_2O), 3.71 (dt, 2H, OC*H*$_2$CH$_2$F), 4.37 (t, 1H, C*H*NH_2), 4.52 (dt, 2H, CH_2F), 4.54 (s, 2H, ArCH_2O), 5.58 (s, 2H, CH_2N), 7.30 (d, 2H, Ar), 7.38 (d, 2H, Ar), 7.96 (s, 1H, Triazol)

TSI-MS: m/z 225, 411 [M+H$^+$], 433, 449, 467, 877

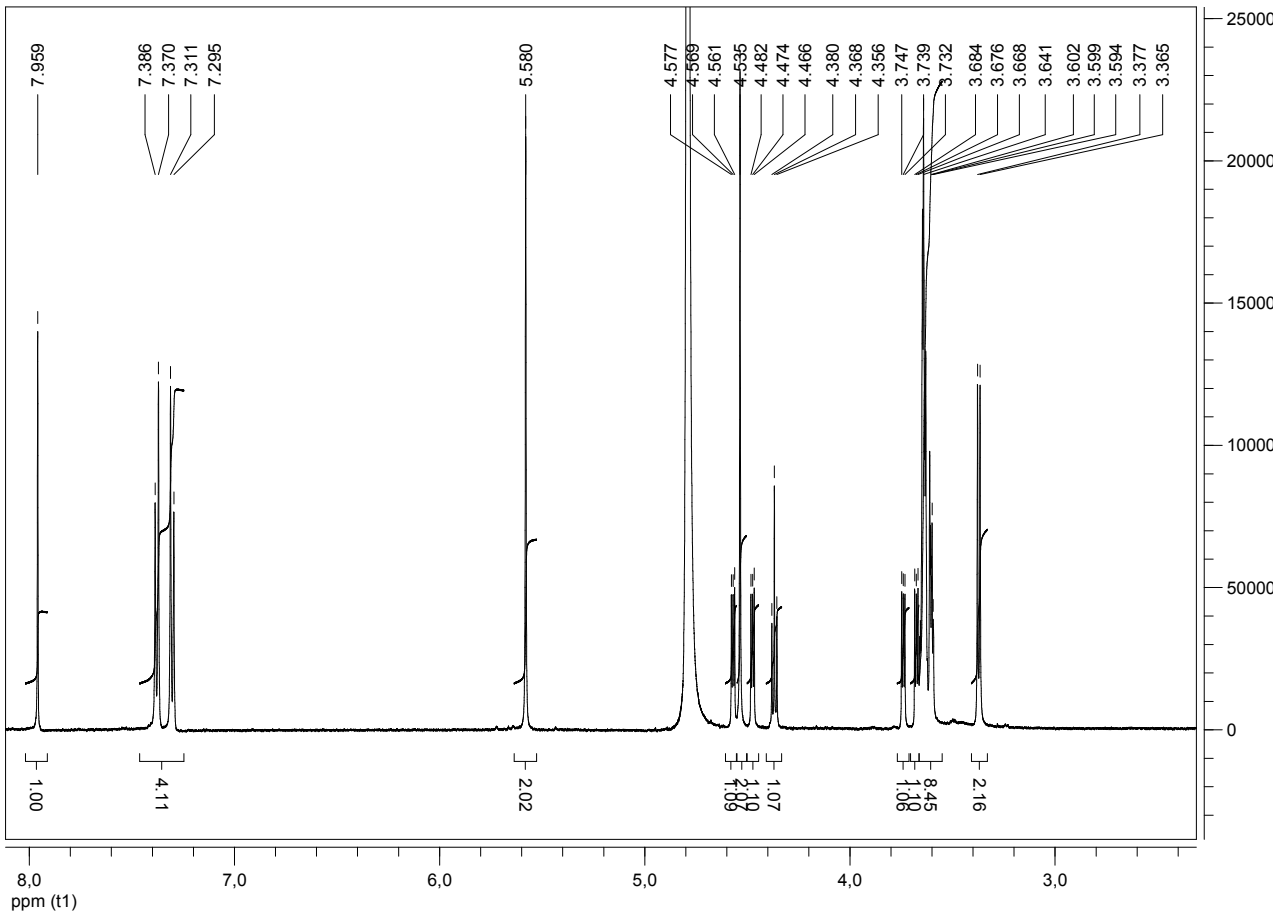


Figure 28 – ^{1}H-NMR compound 25

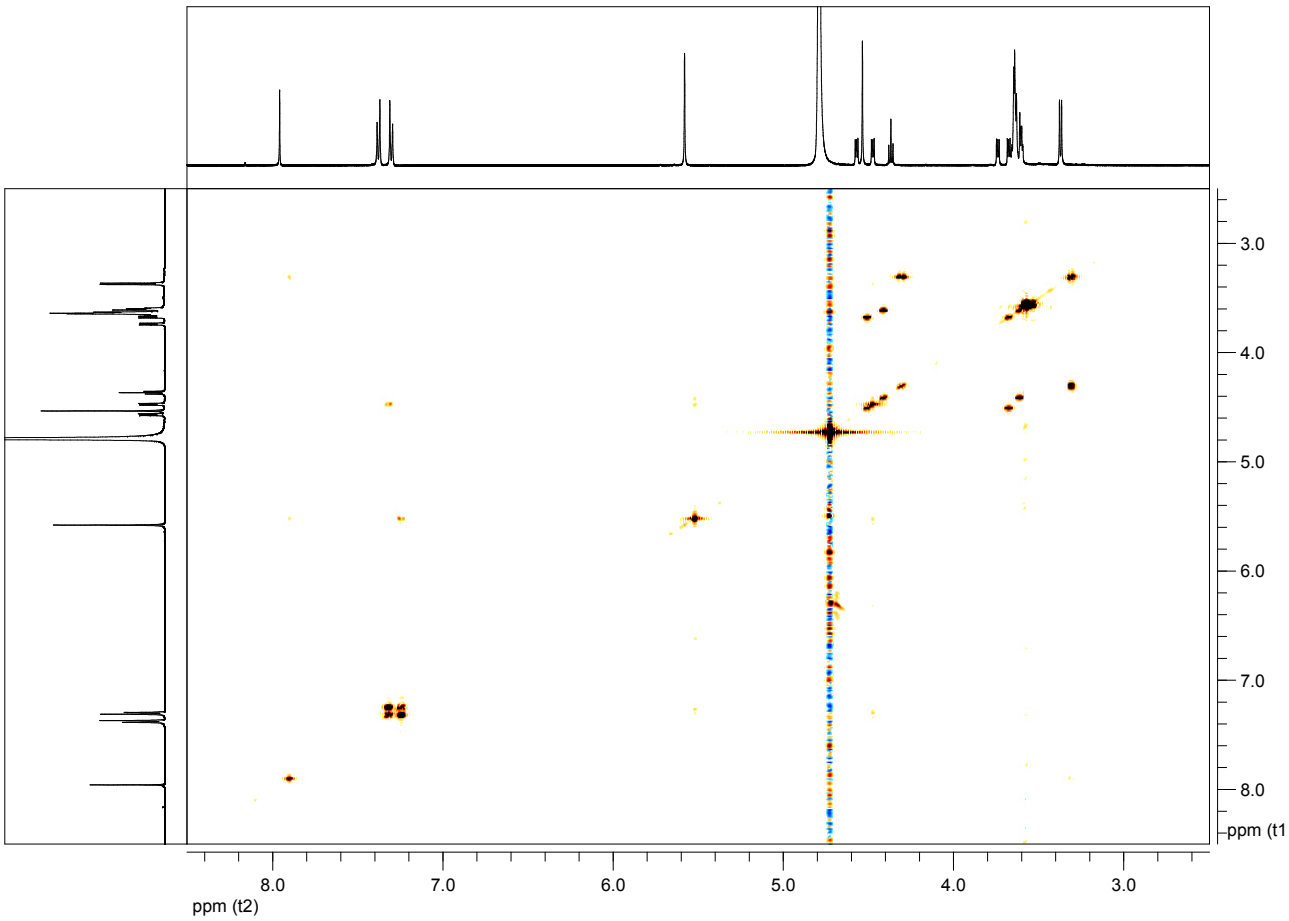
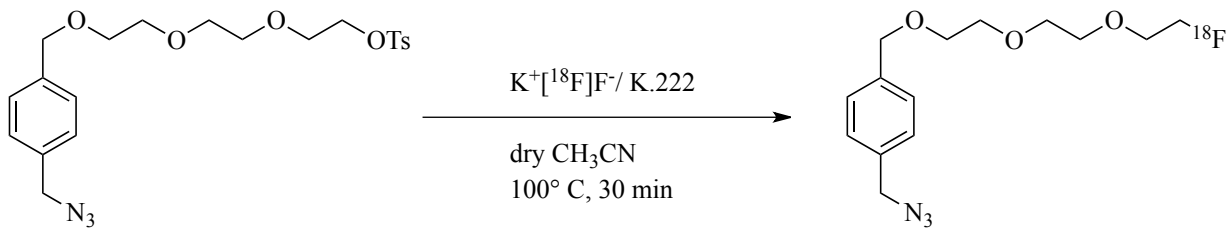


Figure 28 – COSY-NMR compound 25

RADIOCHEMICAL PROCEDURES

1-(azidomethyl)-4-({2-[2-(2-[^{18}F]-fluoroethoxy)ethoxy]ethoxy}methyl)benzene ([^{18}F]1) – Procedure 1



2 **[^{18}F]1** rcy 1%

21 GBq of [^{18}F] fluoride were trapped by passing the target water through a Sep-Pak light QMA cartridge. A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl) was passed through the QMA cartridge to elute the activity in the $K^+[^{18}F]^-$ form directly in the reactor vial. A solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) was added, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **2** (1 mg, 0.002 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 30 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, and deflated. Acetonitrile (0.5 ml), and water (1 ml) were added, and the mixture was passed through two Sep-Pak Al light N cartridges connected in series, previously conditioned with ethanol (10 ml), and water (10 ml), directly into the final vial. The cartridges were washed with water (2 ml), and the volume was collected in the final vial. The obtained final activity was 350 MBq. Analysis by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min flow, 220 nm/ radiochemical detector) revealed that the product **[^{18}F]1** was formed (RCY non corrected for the decay 1%, 52% radiochemical purity).

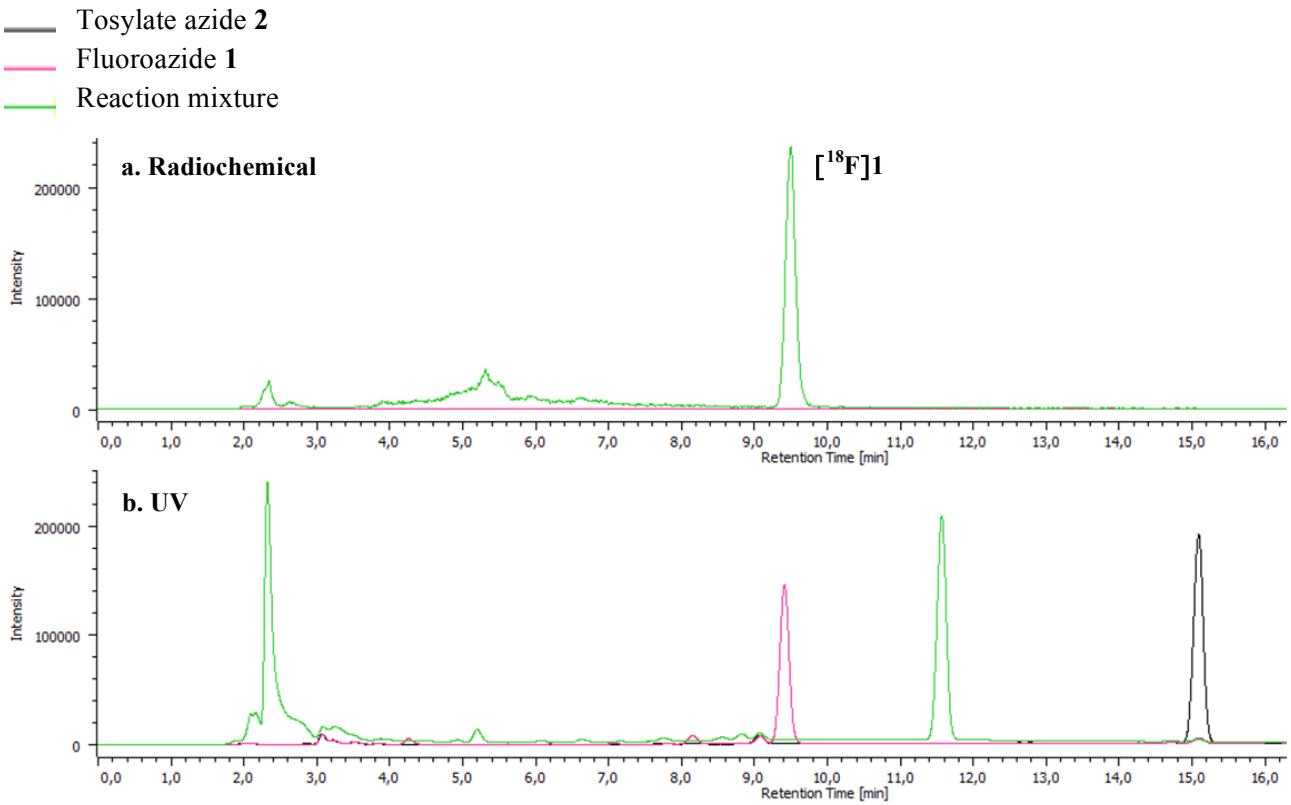


Figure 12

t_R 9.5 min.

Elimination product:

t_R 11.6 min

LC-MS m/z 336

Stability test for compound 2 in reaction conditions (basic conditions)

A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl) and a solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) were mixed in the reactor vial. The mixture was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **2** (1 mg, 0.002 mmol) in anhydrous acetonitrile (1 ml) was added.
Heating cycles of 15 min at various temperature (26, 35, 45, 55, 65, 75° C) under helium pressure (220 KPa) were performed, and the mixture was monitored by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm) to verify stability of compound **2**.

Tosylate azide **2** 15 min 25° C
Tosylate azide **2** 15 min 35° C
Tosylate azide **2** 15 min 45° C
Tosylate azide **2** 15 min 55° C
Tosylate azide **2** 15 min 65° C
Tosylate azide **2** 15 min 75° C

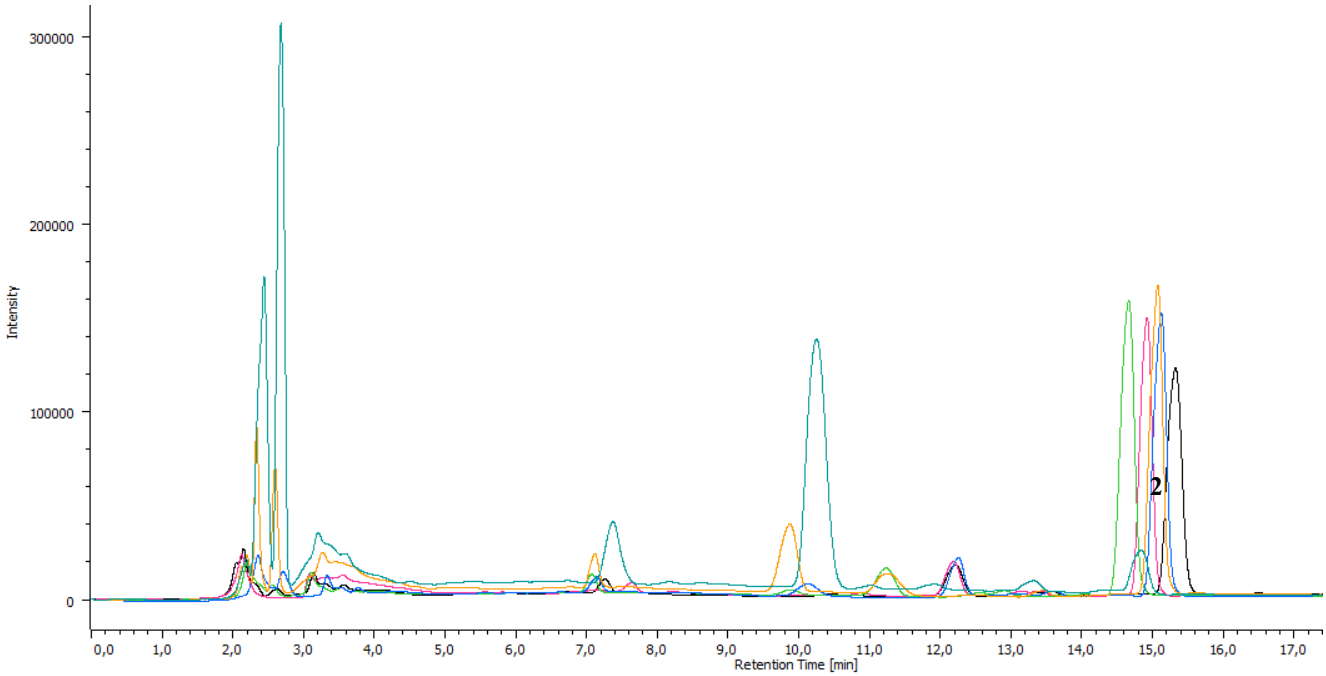
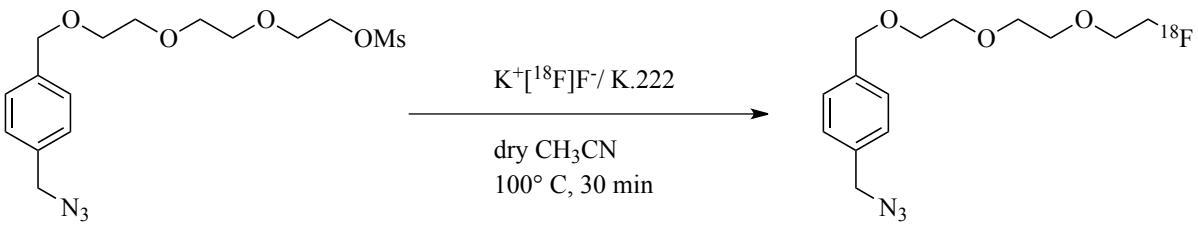


Figure 29

From the temperature of 70° C we could observe significant degradation.

1-(azidomethyl)-4-({2-[2-(2-[^{18}F]-fluoroethoxy)ethoxy]ethoxy}methyl)benzene ([^{18}F]1) – Procedure 2



3 **[^{18}F]1** rcy 57%

20 GBq of [^{18}F] fluoride were trapped by passing the target water through a Sep-Pak light QMA cartridge. A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl) was passed

through the QMA cartridge to elute the activity in the $K^+[^{18}F]^-$ form directly in the reactor vial. A solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) was added, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **3** (10 mg, 0.027 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 20 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, and deflated. Acetonitrile (0.5 ml), and water (1 ml) were added, and the mixture was passed through two Sep-Pak Al light N cartridges connected in series, previously conditioned with ethanol (10 ml), and water (10 ml), directly into the final vial. The cartridges were washed with water (2 ml), and the volume was collected in the final vial. The obtained final activity was 15 GBq. Analysis by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm/ radiochemical detector) revealed that the product **[^{18}F]1** was formed (RCY non corrected for the decay 57%, 74% radiochemical purity). The UV spectra showed the partial degradation of the precursor to afford the hydrolysis product, as confirmed by HPLC injection of compound **22**, and the elimination product.

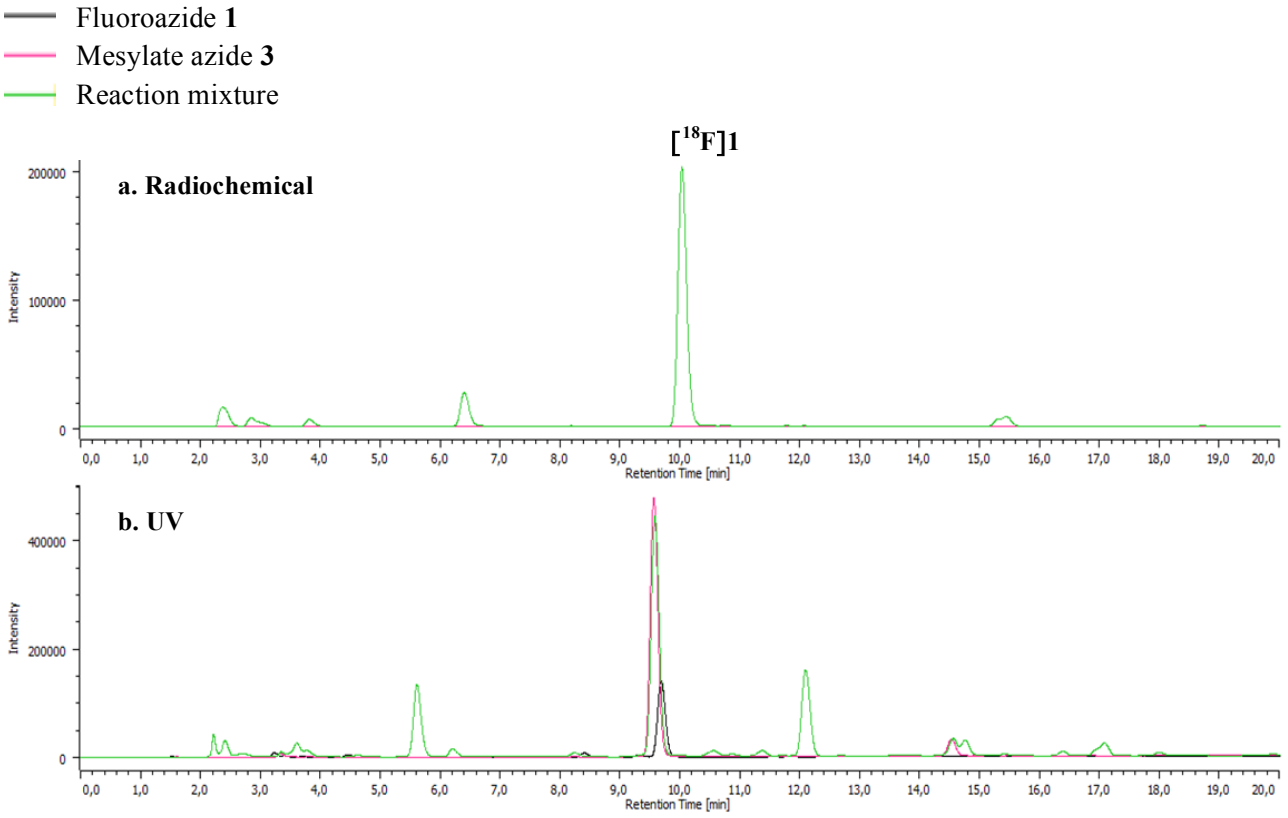


Figure 13

t_R 10.0 min.

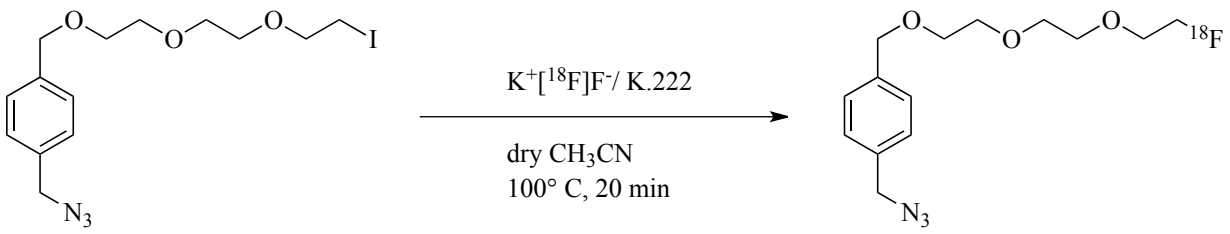
Hydrolysis product:

t_R 5.6 min.

Elimination product:

t_R 12.1 min.

1-(azidomethyl)-4-({2-[2-(2-[^{18}F]-fluoroethoxy)ethoxy]ethoxy}methyl)benzene ([^{18}F]1) – Procedure 3



5 **[^{18}F]1** rcy 61%

20 GBq of [^{18}F] fluoride were trapped by passing the target water through a Sep-Pak light QMA cartridge. A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl) was passed through the QMA cartridge to elute the activity in the $K^+[^{18}F]^-$ form directly in the reactor vial. A solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) was added, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **5** (10 mg, 0.025 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 20 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, and deflated. Acetonitrile (0.5 ml), and water (1 ml) were added, and the mixture was passed through two Sep-Pak Al light N cartridges connected in series, previously conditioned with ethanol (10 ml), and water (10 ml), directly into the final vial. The cartridges were washed with water (2 ml), and the volume was collected in the final vial. The obtained final activity was 14.4 GBq. Analysis by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm/ radiochemical detector) revealed that the product **[^{18}F]1** was formed (RCY non corrected for the decay 61%, 85% radiochemical purity). The UV spectra showed the precursor complete degradation to afford the hydrolysis product.

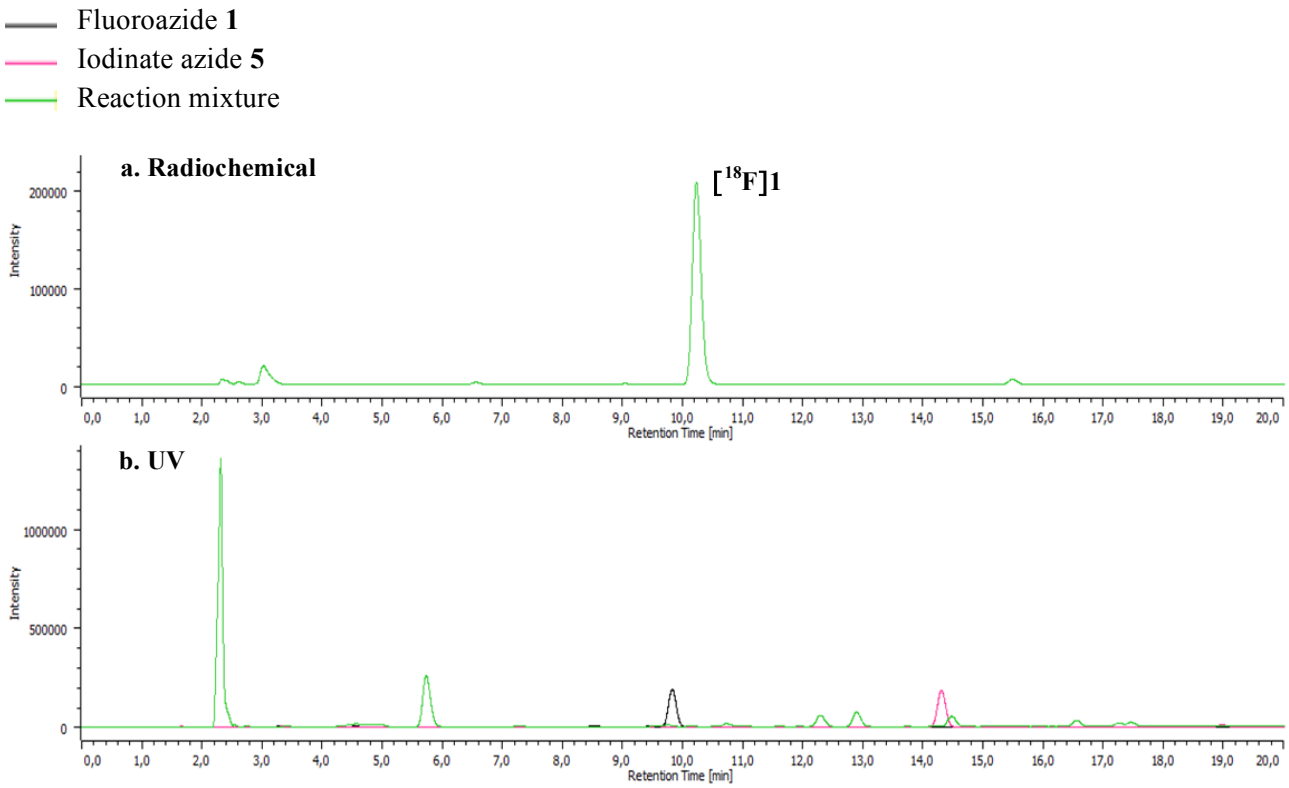


Figure 14

t_R 10.2 min.

Hydrolysis product:

t_R 5.7 min.

Stability test for compound 5 in heating conditions

0.2 mg (0.0005 mmol) of compound **5** were suspended in 1 ml anhydrous acetonitrile and heated to 100° C for 20 minutes. The mixture composition was monitored by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm) at interval of 5 min.

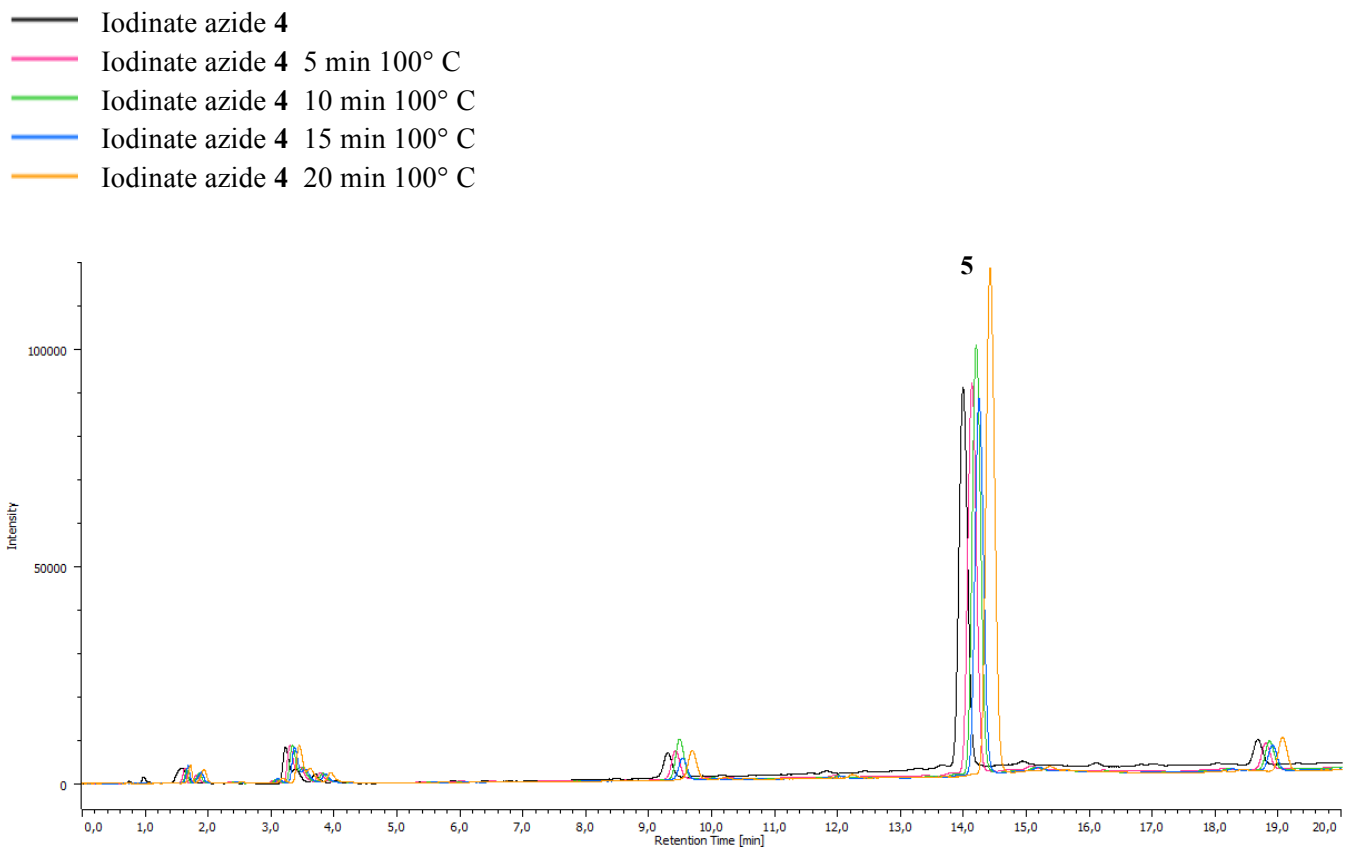


Figure 30

Figure 30 show the stability of compound **5** to temperature.

Stability test for compound 5 in reaction conditions (basic conditions)

A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl), and a solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) were mixted in the reactor vial. The solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **5** (0.2 mg, 0.0005 mmol) in anhydrous acetonitrile (1 ml) was added. The mixture was heated 10 min at 70° C, 10 min at 80° C, 5 min at 100° C, 10 min at 100° C.

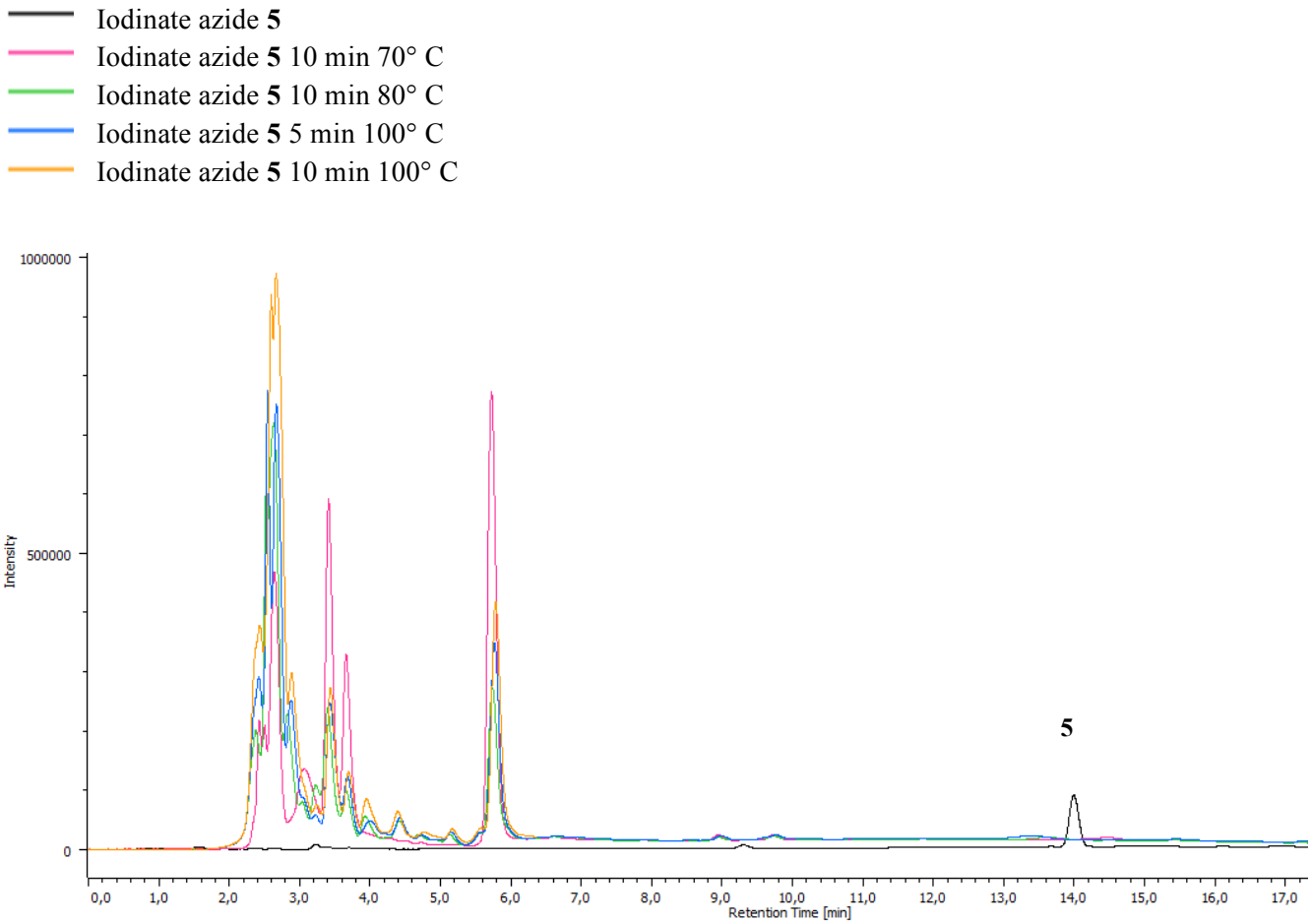


Figure 31

Full degradation seemed to occur yet at 70° C.

These results were in contrast with the fact that at 100° C we obtained a high radiolabelling yield, so the same test was performed at ambient temperature.

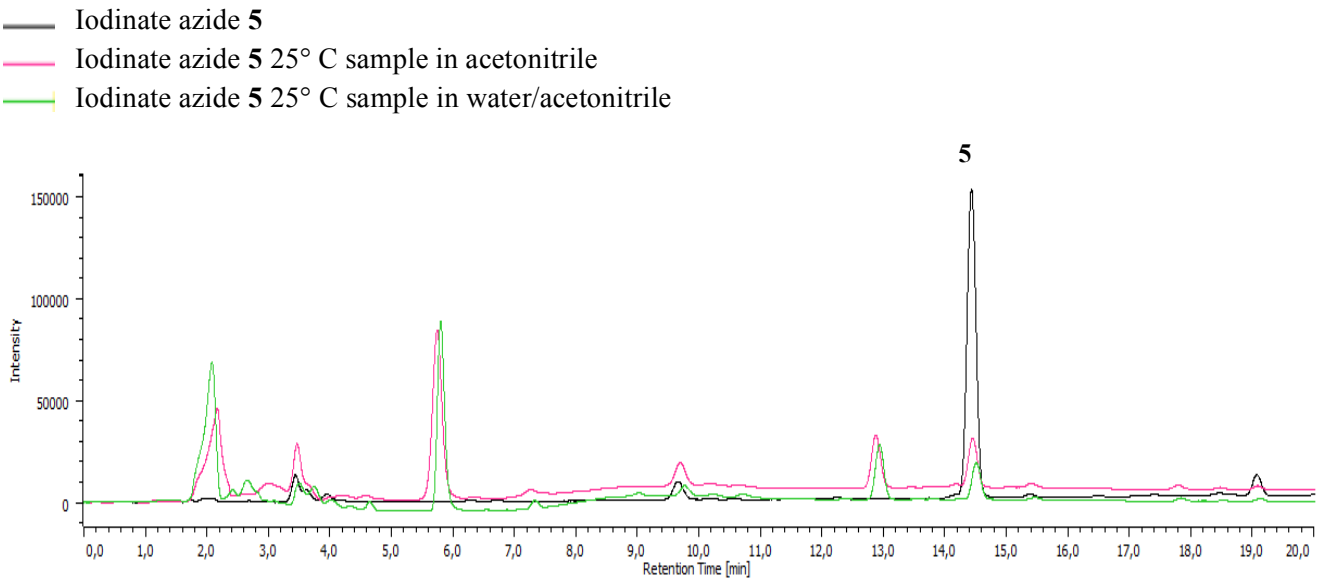


Figure 32

Immediately monitoring of the mixture diluted in 100% acetonitrile or a mixture water/acetonitrile showed again almost complete degradation. This fact confirmed that precursor **5** hydrolyzes immediately when diluted in water in basic environment.

Stability test for compound 5 in basic conditions using $TBAHCO_3$

0.25 ml of a solution of compound **5** (0.2 mg, 0.0005 mmol) in acetonitrile are mixed to 0.25 ml of a solution of $TBAHCO_3$ 0.075 M in water.
0.25 ml of a solution of compound **5** (0.2 mg, 0.0005 mmol) in acetonitrile are mixed to 0.25 ml of a solution of $TBAHCO_3$ 0.075 M in water passed through a Sep-Pak QMA cartridge.
The obtained mixtures were analyzed by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm).

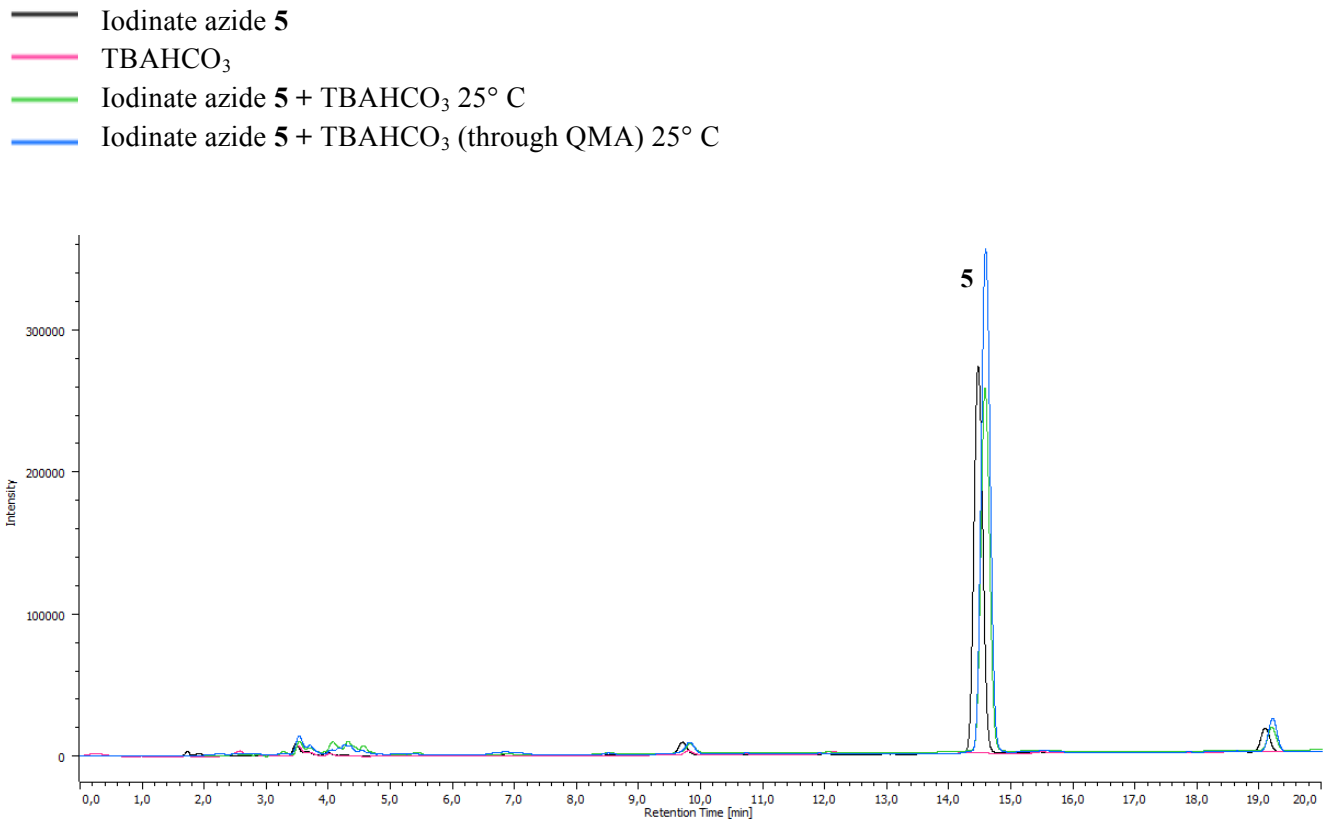
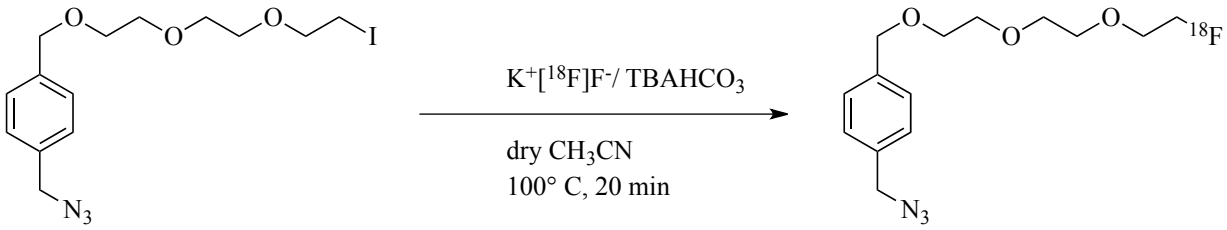


Figure 33

Compound **5** not degraded.

1-(azidomethyl)-4-({2-[2-(2-[^{18}F]-fluoroethoxy)ethoxy]ethoxy}methyl)benzene ([^{18}F]1) – Procedure 4



5 **[^{18}F]1** rcy 57%

17 GBq of [^{18}F] fluoride were trapped by passing the target water through a Sep-Pak light QMA cartridge. A solution of tetrabutylammonium hydrogen carbonate (0.5 ml 0.075 M $TBAHCO_3$ in water) was passed through the QMA cartridge to elute the activity in the $K^+[^{18}F]^-$ form directly in the reactor vial. 1 ml acetonitrile was added, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **5** (10 mg, 0.025 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 20 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, and deflated. Acetonitrile (0.5 ml), and water (1 ml) were added, and the mixture was passed through two Sep-Pak Al light N cartridges connected in series, previously conditioned with ethanol (10 ml), and water (10 ml), directly into the final vial. The cartridges were washed with water (2 ml), and the volume was collected in the final vial. The obtained final activity was 10.2 GBq. Analysis by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm/ radiochemical detector) revealed that the product **[^{18}F]1** was formed (RCY non corrected for the decay 57%, 95% radiochemical purity). The UV spectra showed the precursor degradation to afford a complex mixture.

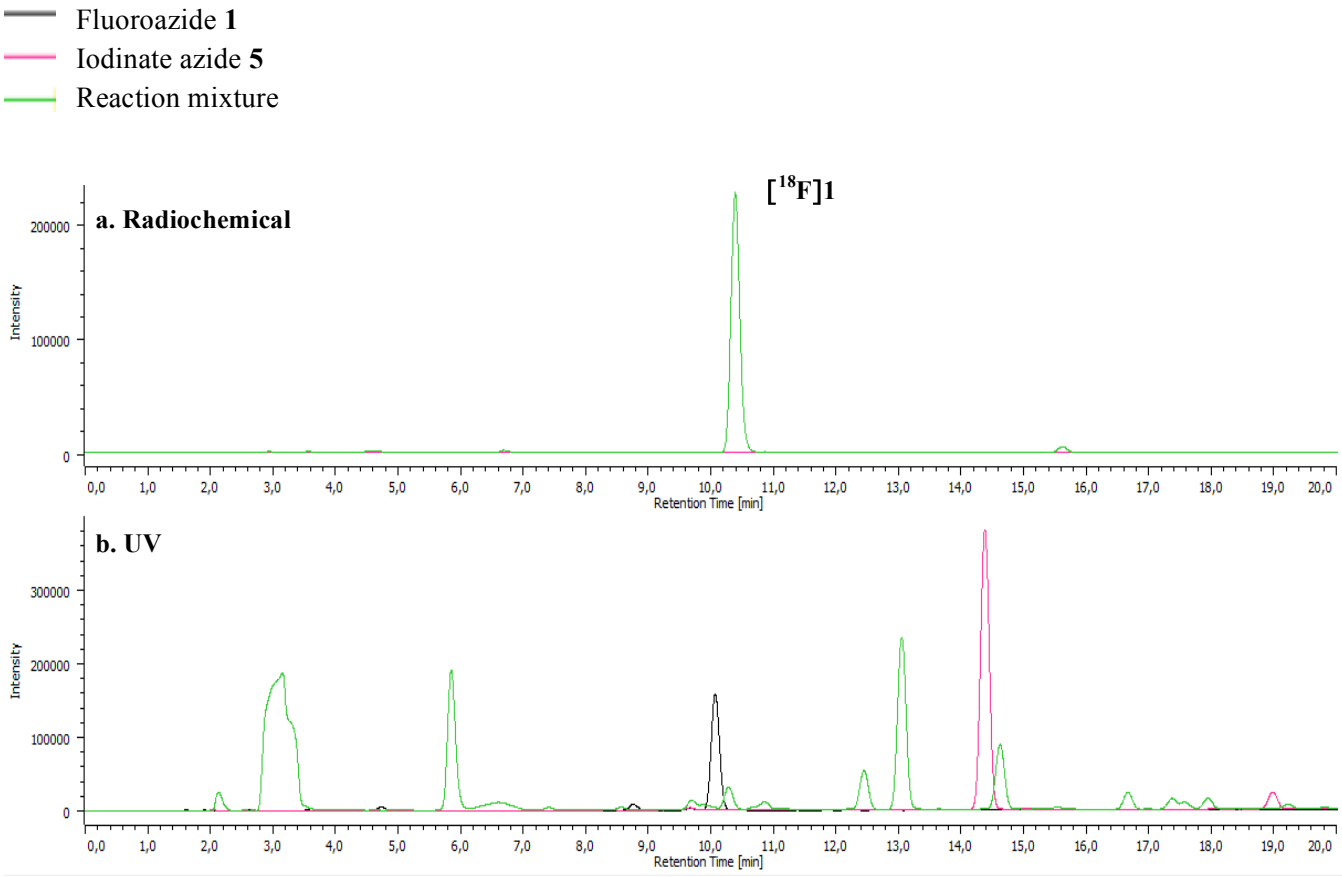


Figure 15

t_R 10.4 min.

Stability test for compound 5 in buffered conditions

0.5 ml of PBS pH 6.0 (50 mM) were added to 3.75 mg $KHCO_3$. The measured pH was 6.4.
0.5 ml of PBS pH 6.0 (50 mM) were added to 2.51 mg K_2CO_3. The measured pH was 6.8.

A solution of tetrabutylammonium hydrogen carbonate (0.5 ml 0.075 M $TBAHCO_3$ in water) and 1 ml acetonitrile were mixed together in the reaction vial, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **5** (2.5 mg, 0.006 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 20 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, and 0.5 ml of PBS pH 6.0 (50 mM) were added. The mixture was analyzed by

RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm).

A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl) and a solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) were mixed together in the reaction vial, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **5** (2.5 mg, 0.006 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 20 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, and 0.5 ml of PBS pH 6.0 (50 mM) were added. The mixture was analyzed by RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm).

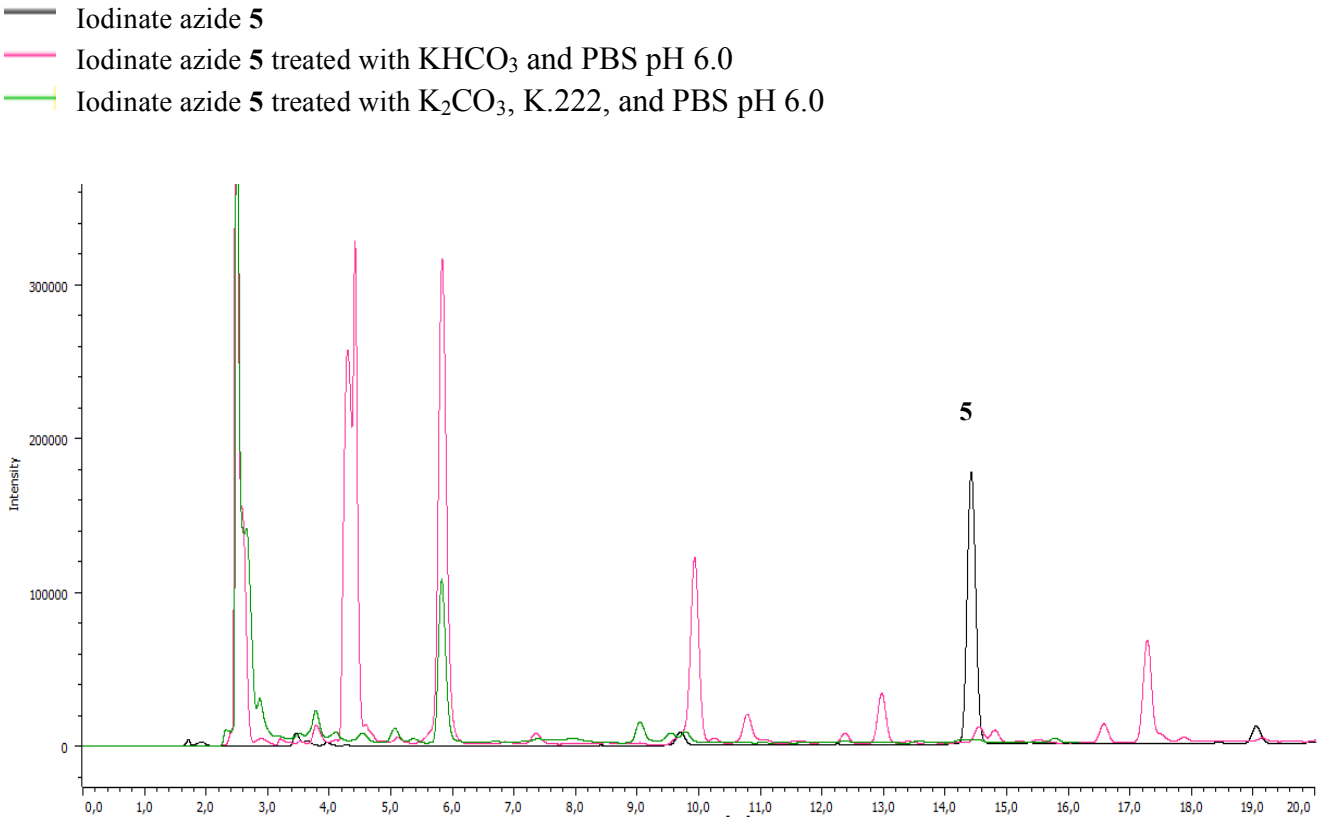


Figure 34

Complete degradation of iodinate precursor **5** was observed.

Purification of 1-(azidomethyl)-4-({2-[2-(2-[^{18}F] fluoroethoxy)ethoxy]ethoxy}methyl)benzene ([^{18}F]1) – Method 1

A solution of crude compound **[^{18}F]1** (starting [^{18}F]fluoride activity 33 GBq) diluted in water/acetonitrile 9:1 (18 ml) was passed through a Sep-Pak tC18 Plus cartridge previously conditioned with 10 ml ethanol, and 10 ml water. The cartridge was washed with a solution of water/acetonitrile 8:2 (10 ml), and eluted with 3 ml acetonitrile, to afford the pure product **[^{18}F]1** (18 GBq, 51% RCY non-corrected for the decay, 93% radiochemical purity), as confirmed by RP-HPLC analysis (XTerra C18 column, water/acetonitrile gradient from 60:40 to 20:80 in 20 min, 1 ml/min, 220 nm/ radiochemical detector).

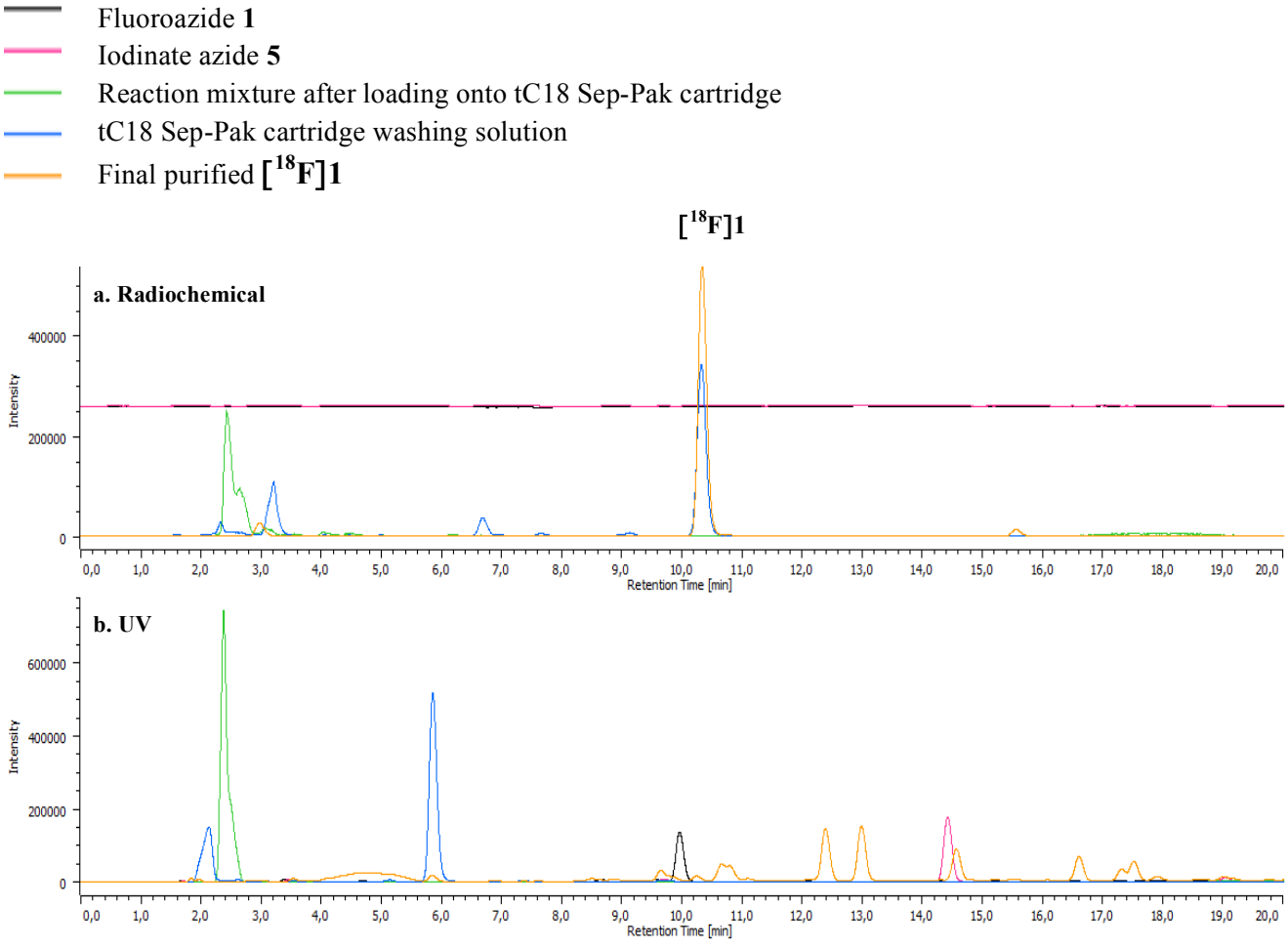


Figure 16

t_R 10.4 min.

Purification of 1-(azidomethyl)-4-({2-[2-(2-[^{18}F]fluoroethoxy)ethoxy]ethoxy}methyl)benzene ([^{18}F]1) – Method 2

RP-HPLC method tested on an XBridge PREP C18 (10x150 mm, 5 µm) column.

Elution was performed from 0.5 min isocratic 50:50 water/acetonitrile, then gradient to 20:80 water/acetonitrile in 20 min, then gradient to 10:90 water/acetonitrile in 10 min.
Injected: alcohol azide **22** (t_R 8.2 min), iodinate azide **5** (t_R 11.2 min), fluorinated azide **1**(t_R 14.3 min). Separation and peak thickness improved if diluted in the maximum percentage of water then acetonitrile (in this case water was 99%).

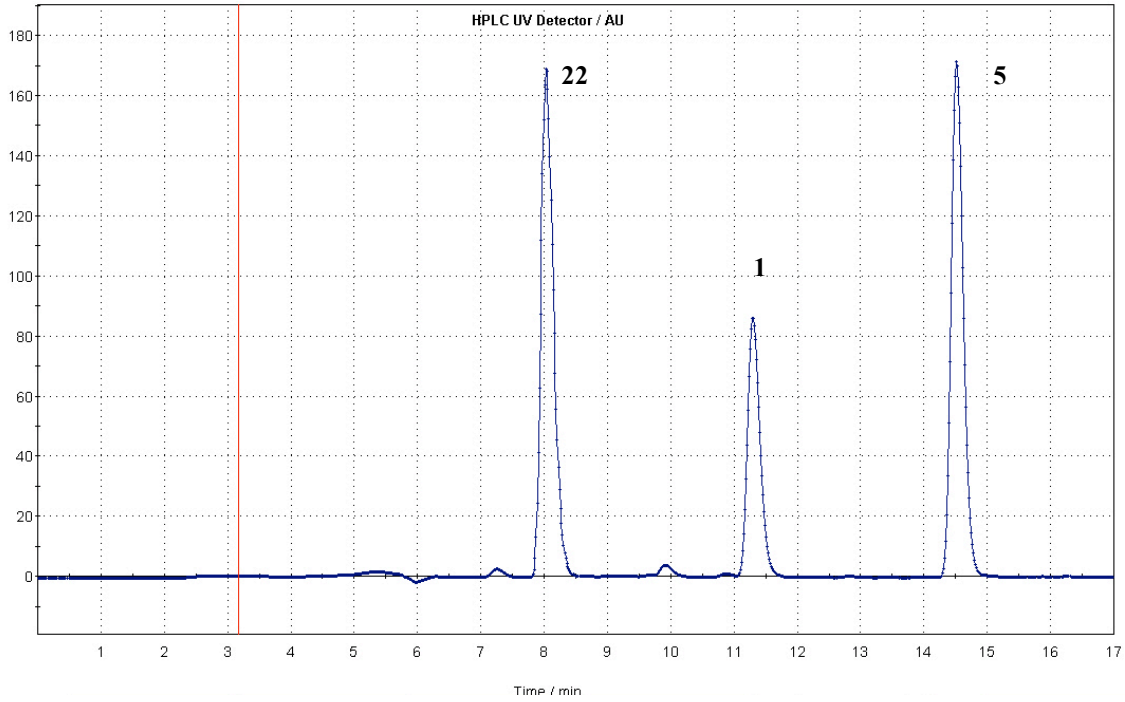


Figure 35

A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl) and a solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) were mixed together in the reaction vial, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **5** (1 mg, 0.003 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 20 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, then fluorinated azide **1** (0.3 ml in acetonitrile) was added to the mixture, which was diluted with water (1.2 ml) and injected in RP-HPLC.

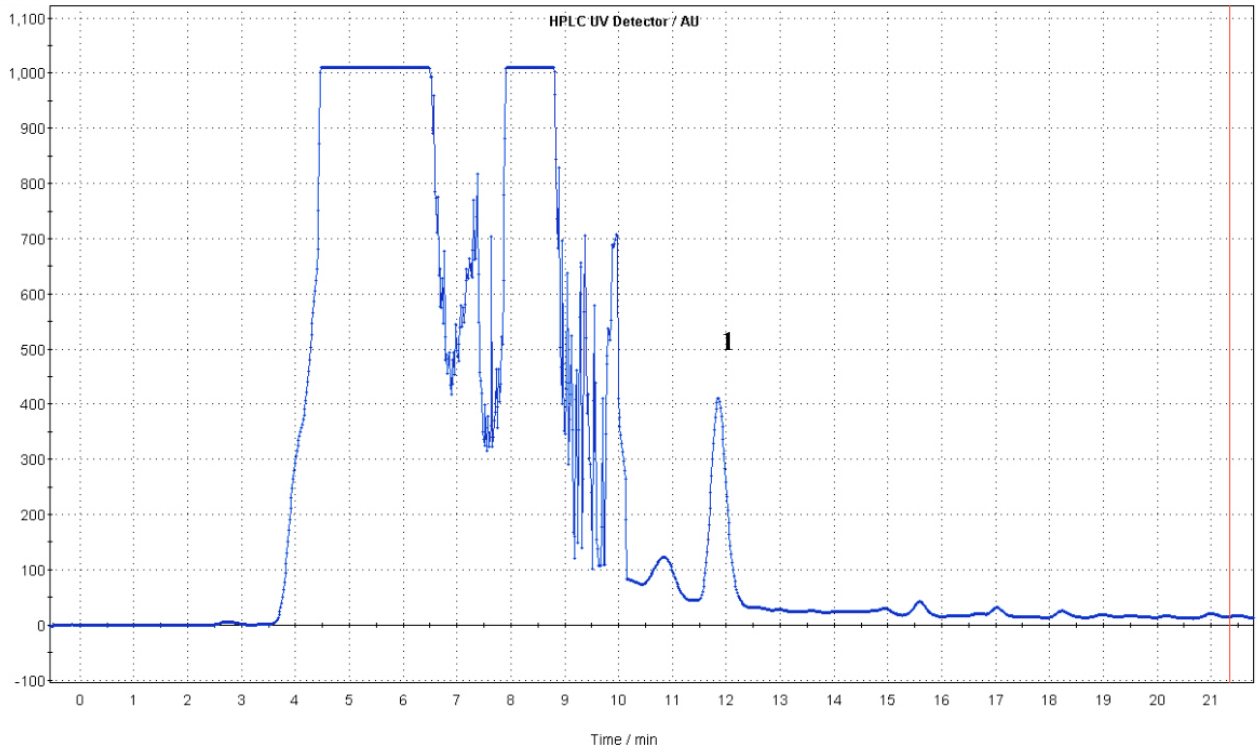


Figure 36

The product was collected in 1.7 ml elution solvent.

16 GBq of [^{18}F] fluoride were trapped by passing the target water through a Sep-Pak light QMA cartridge. A solution of potassium carbonate (2.51 mg, 0.018 mmol) in water (500 µl) was passed through the QMA cartridge to elute the activity in the $K^+[^{18}F]^-$ form directly in the reactor vial. A solution of kryptofix.222 (15 mg, 0.04 mmol) in acetonitrile (1 ml) was added, and the solvent was azeotropically distilled at 85° C, under helium stream. The reactor was cooled-down to 30° C, and a solution of compound **5** (1 mg, 0.003 mmol) in anhydrous acetonitrile (1 ml) was added. The reaction mixture was stirred at 100° C for 20 min under helium pressure (220 KPa). The reactor was cooled-down to room temperature, and deflated. The mixture was diluted with 1.4 ml water and injected to be purified by semipreparative RP-HPLC (Figure 28).

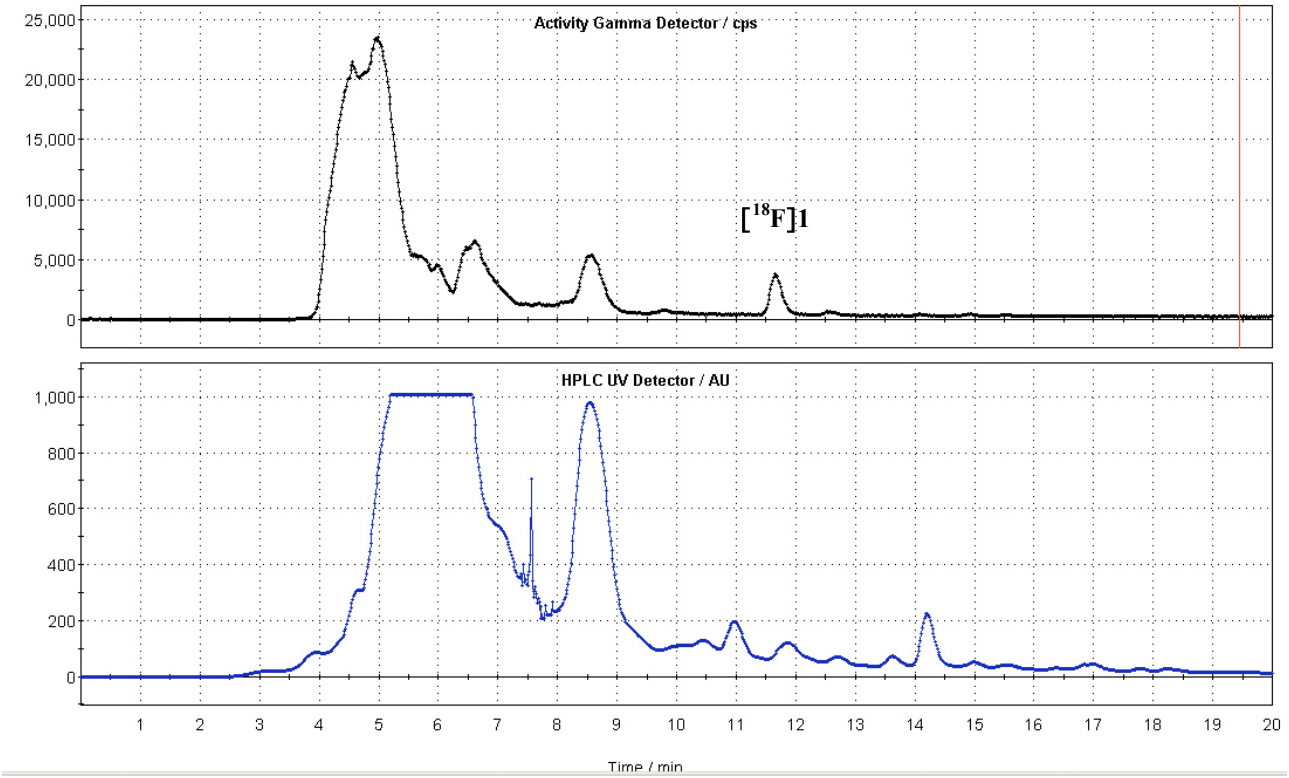
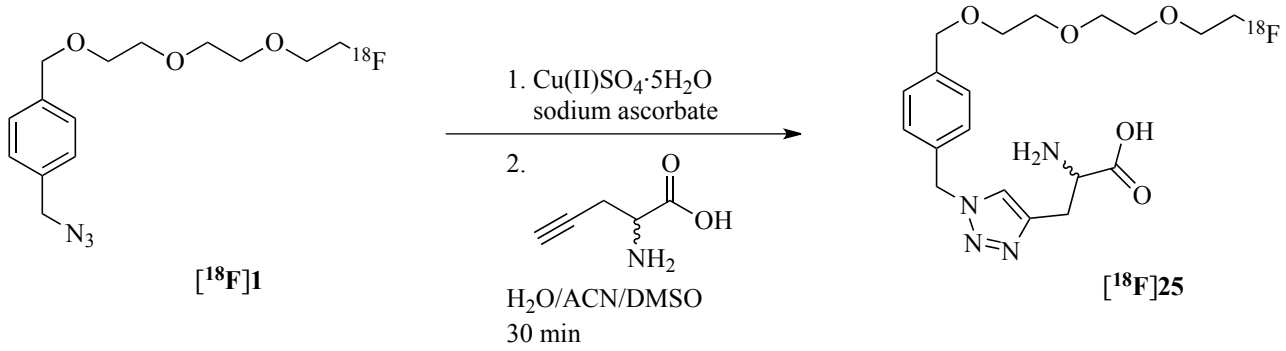


Figure 37

1 mg of precursor was demonstrated to be inadequate. The purification was not effective even at this low amount.

3-{1-[4-({2-[2-(2-[^{18}F]fluoroethoxy)ethoxy]ethoxy}methyl)benzyl]-1*H*-1,2,3-triazol-4-yl}alanine ([^{18}F]25)



[^{18}F]1 [^{18}F]25

Starting [^{18}F]fluoride activity 34 GBq.

[^{18}F]1 was produced by the method previously described in this experimental session (see **[^{18}F]1** - Procedure 3), purified by tC18 Sep-Pak cartridge (see Purification of **[^{18}F]1** - Method 1), and collected in 0.5 ml acetonitrile. To this preparation 0.2 ml dimethylsulfoxyde were added.

To a fresh-prepared solution of copper(II) sulfate pentahydrate (6.7 mg, 0.027 mmol, 1.5 eq) in 50 µl water in the reaction vial was added a fresh-prepared solution of sodium ascorbate (16.0 mg, 0.081 mmol, 4.5 eq) in 300 µl water, under Helium. After 2 min a solution of propargylglycine (2 mg, 0.018 mmol, 1 eq) in 100 µl PBS pH 6.0 (50 mM) was added. After 2 min the solution of compound **[^{18}F]1** was added. The reaction mixture was stirred at room temperature for 30 min. The reaction was quenched with 1 M hydrochloric acid (0.5 ml), and the mixture was diluted with water (5 ml), and collected in the final vial. The final activity was 11. 6 GBq.
Total time synthesis was 1 h 20 min.
Analytical RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 90:10 to 30:70 in 24 min, 1 ml/min, 220 nm, radiochemical detector) revealed that 52% conversion was obtained from **[^{18}F]1**, to achieved crude product **[^{18}F]25** (18% RCY non-corrected for the decay, 28% RCY corrected for the decay).

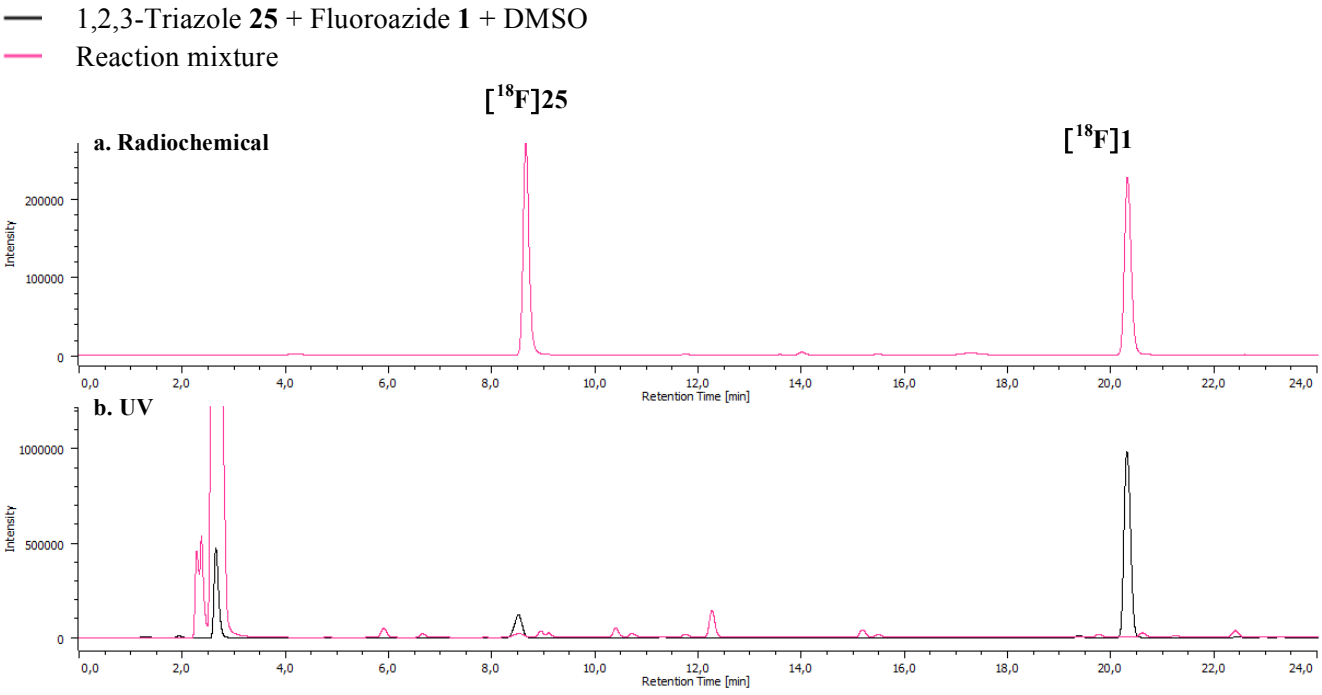


Figure 17

t_R 8.7 min

Purification of 3-{1-[4-({2-[2-(2-[^{18}F]fluoroethoxy)ethoxy]ethoxy}methyl)benzyl]-1*H*-1,2,3-triazol-4-yl}alanine ([^{18}F]25)

Starting [^{18}F]fluoride activity was 29 GBq.

[^{18}F]1 was produced by the method previously described in this experimental session (see **[^{18}F]1** - Procedure 3), purified by tC18 Sep-Pak cartridge (see Purification of **[^{18}F]1** - Method 1), and collected in 0.5 ml acetonitrile. To this preparation 0.2 ml dimethylsulfoxyde were added.

To a fresh-prepared solution of copper(II) sulfate pentahydrate (6.7 mg, 0.027 mmol, 1.5 eq) in 50 µl water in the reaction vial was added a fresh-prepared solution of sodium ascorbate (16.0 mg, 0.081 mmol, 4.5 eq) in 300 µl water, under Helium. After 2 min a solution of propargylglycine (2 mg, 0.018 mmol, 1 eq) in 100 µl PBS pH 6.0 (50 mM) was added. After 2 min the solution of compound **[^{18}F]1** was added. The reaction mixture was stirred at room temperature for 30 min. The reaction was quenched with 1 M hydrochloric acid (1.2 ml), and semipreparative RP-HPLC was performed: XBridge PREP C18 column, isocratic 0.5 min 90:10 water (+0,1% TFA)/acetonitrile (+0,1% TFA), then gradient to 30:70 water (+0,1% TFA)/acetonitrile (+0,1% TFA) in 24 min, then gradient to 10:90 water (+0,1% TFA)/acetonitrile (+0,1% TFA) in 10 min, 2ml/min flow, 220 nm, radiochemical detector.

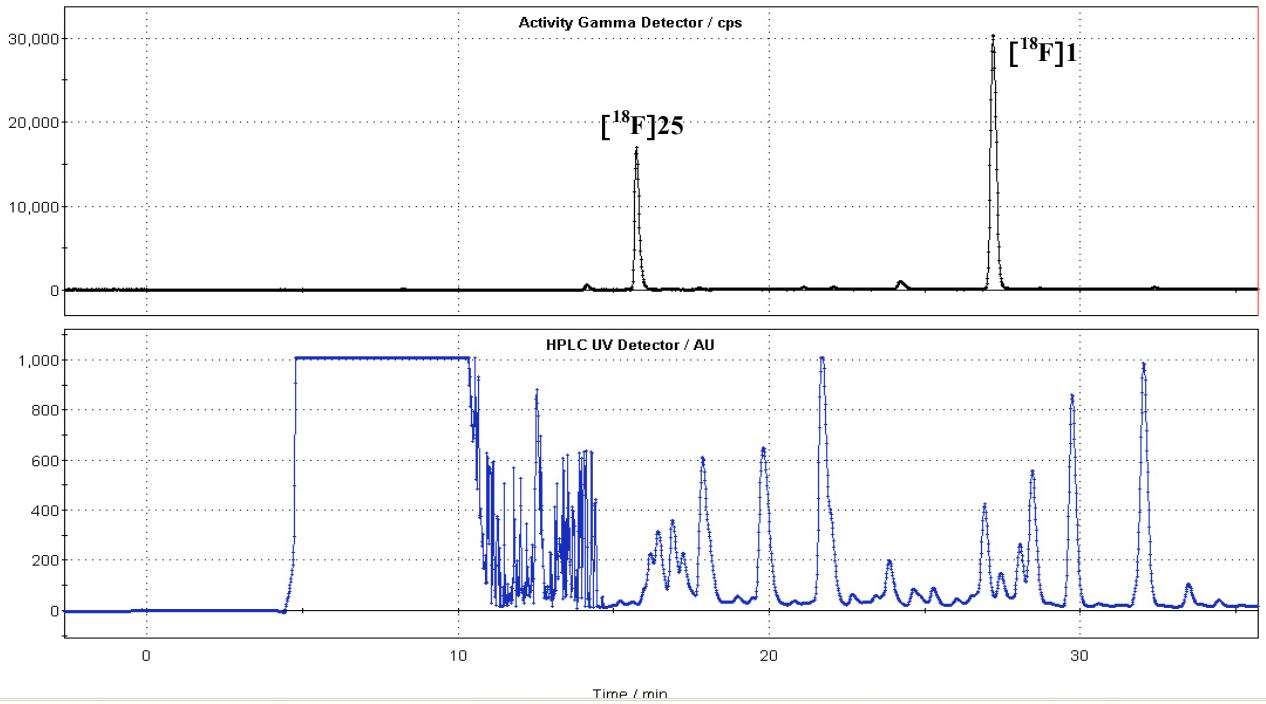


Figure 18

t_R 15.3 min

Total synthesis time was 1 h 30 min.

The pure product **[^{18}F]25** was collected in the final vial and analyzed by analytical RP-HPLC (XTerra C18 column, water/acetonitrile gradient from 90:10 to 30:70 in 24 min, 1 ml/min flow, 220 nm, radiochemical detector).

The final activity of pure product **[^{18}F]25** was 870 MBq (3% RCY non-corrected for the decay, 100% radiochemical purity, 5% RCY corrected for the decay).

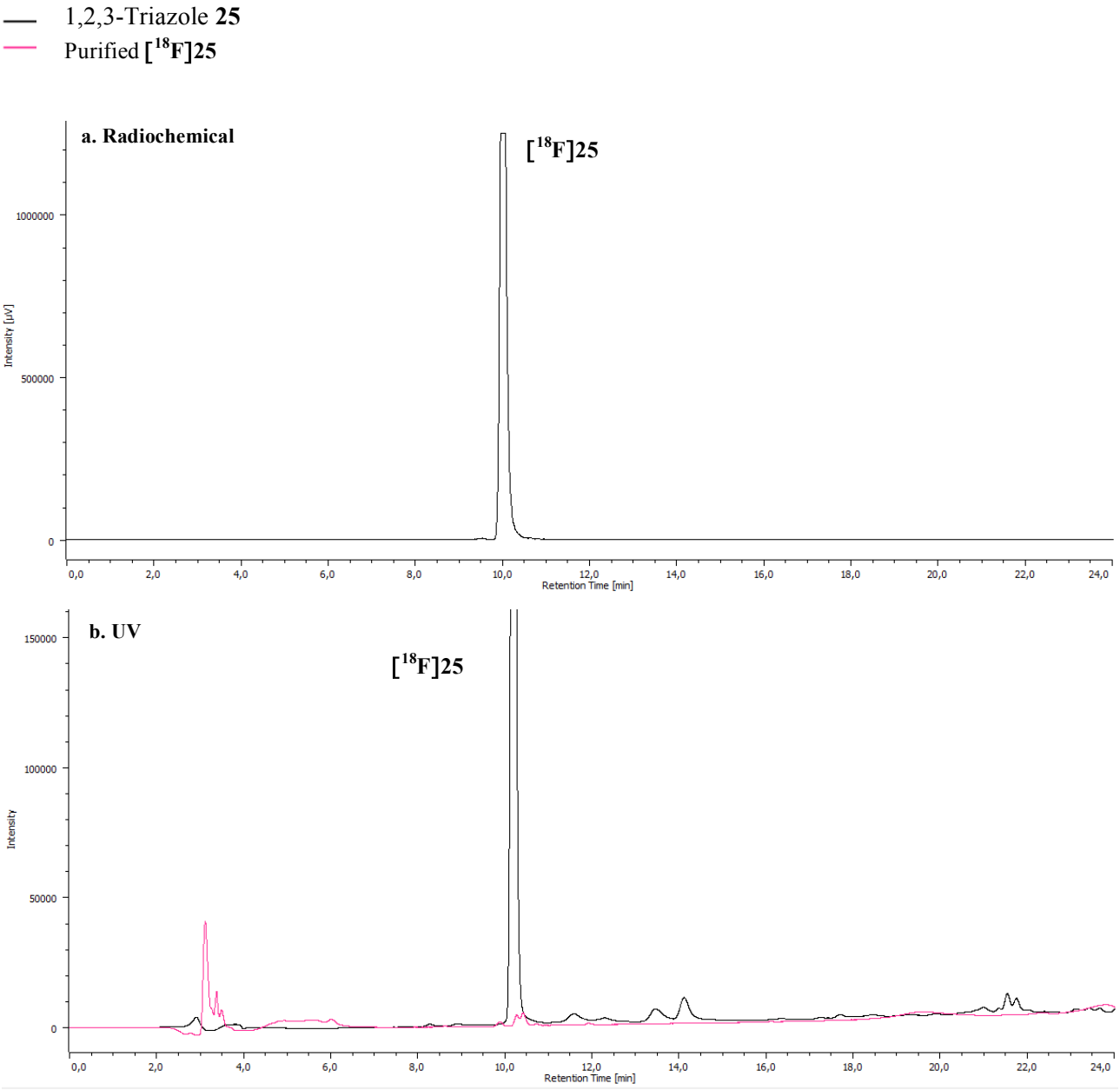


Figure 19

Conversion and total activity were less than expected.

Mechanical problems during collection of semipreparative fraction were effective.

ACKNOWLEDGEMENTS

I thank my tutor, Dr. Sergio Todde, who proposed me to undertake PhD studies and supported this project, and Prof. Patrizia Ferraboschi, who daily sustained chemical synthesis work.

I also thank Stefano Stucchi and Samuele Ciceri for their precious aid concerning technical aspects of radiochemical and chemical activities, and people of both labs that supported me and demonstrated kindness and friendship during these years (Liliana Giuliano, Elia Anna Turolla, and Benedetta Guidi).

My thanks are also addressed to Prof. Fulvio Magni and Clizia Chinello for mass analysis, and Nicola Colombo for IR analysis.

REFERENCES

(1) J. Smith T. A. D., Labelled Compd. Radiopharm **2012**, *55*, 281-288

(2) Chansaenpak K., Wang M., Wu Z., Zaman R., Li Z., Gabbaï F. P., Chem. Commun. **2015**, *51*, 12439-12442

(3) Coenen H. H. *Ernst Schering Res. Found. Workshop*, **2007**, *62*, 15-50

(4) Roeda D., Dolle F. *Chemistry of Molecular Imaging*, **2015**, 55-77

(5) Pretze M., Pietzsch D., Mamat C. *Molecules*, **2013**, *18*, 8618-8665

(6) Banister S., Roeda D., Dollé F., Kassiou M. *Current Radiopharmaceuticals*, **2010**, *3*, 68-80

(7) Moses J. E., Moorhouse A. D. Chem. Soc. Rev. **2007**, *36*, 1249-1262

(8) Nandivada H., Jiang X., Lahann J. *Adv. Mater.*, **2007**, *19*, 2197-2208

(9) Golas P. L., Matyjaszewski K. *Chem. Soc. Rev.*, **2010**, *39*, 1338-1354

(10) El-Sagheer A. H., *Brown T., Chem. Soc. Rev.*, **2010**, *39*, 1302-1315

(11) Gramlich P. M., Wirges C. T., Manetto A., Carell T. *Angew. Chem., Int. Ed.*, **2008**, *47*, 8350-8358

(12) Laughlin S. T., Bertozzi C. R *Proc. Natl. Acad. Sci. U.S.A.*, **2009**, 106, 12-17

(13) Yamada T., Peng C. G., Matsuda S., Addepalli H., K. Jayaprakash K. N., Alam M. R., Mills K., Maier M. A., Charisse K., Sekine M., Manoharan M., Rajeev K. G. *J. Org. Chem.*, **2011**, *76*, 1198-1211

(14) Kolb H. C., Sharpless K. B. *Drug Discovery Today*, **2003**, *8*, 1128-1137

(15) Marik J., Sutcliffe J. L. *Tetrahedron Lett.*, **2006**, *47*, 6681-6684

(16) Glaser M., Aarstad E. *Bioconjugate Chem.*, **2007**, *18*, 989-993

(17) Rostovtsev V. V., Green L. G., Fokin V. V., Sharpless K. B., *Angew. Chem. Int. Ed.*, **2002**, *41*, 2596-2599

(18) Gierlich J., Burley G. A., Gramlich P. M. E., Hammond D. M., Carell T. *Org. Lett.*, **2006**, *8*, 3639-3642

(19) Burrows C. J., Muller J. G. *Chem. Rev.*, **1998**, *98*, 1109-1152

(20) Gaetke L. M., Chow C. K. *Toxicology*, **2003**, *189*, 147-163

(21) Besanceney-Webler C., Jiang H., Zheng T., Feng L., Soriano del Amo D., Wang W., Klivansky L. M., Marlow F. L., Liu Y., Wu P. *Angew. Chem. Int. Ed.* **2011**, *50*, 8051-8056

(22) Boren B. C., Narayan S., Rasmussen L. K., Zhang L., Zhao H., Lin Z., Jia G., Fokin V. V. *J. Am. Chem. Soc.* **2008**, *130*, 8923-8930

(23) Rasolofonjatovo E., Theeramunkong S., Bouriaud A., Kolodych S., Chaumontet M., Taran F. *Org. Lett.* **2013**, *15*, 4698-4701

(24) Golas P. L., Tsarevsky N. V., Sumerlin B. S., Matyjaszewski K., *Macromolecules*, **2006**, *39*, 6451–6457

(25) Salam N., Sinha A., Roy A. S., Mondal P., Jana N. R., Islam S. M., *RSC Adv.*, **2014**, *4*, 10001-10012

(26) Powers A. R., Ghiviriga, I., Abboud K. A., Veige A. S. *Dalton Transactions*, **2015**, *44*, 14747-14752

(27) Saba B., Sharma S., Sawant D., Kundu B., *Synlett*, **2007**, 1591-1594

(28) Smith C. D., Greaney M. F. *Org. Lett.,* **2013**, *15*, 4826-4829

(29) Becer C. R., Hoogenboom R., Schubert U. S. *Angew. Chem. Int. Ed.* **2009**, *48*, 2-11

(30) Hein J. E., Fokin V. V. *Chem Soc Rev.* **2010**, *39*, 1302–1315

(31) Mykhalichko B. M., Temkin O. N., Mys'kiv G. M. *Russ. Chem. Rev.*, **2000**, *69*, 957–984

(32) Bock V. D., Hiemstra H., van Maarseveen J. H. *Eur. J. Org. Chem.*, **2006**, 51–68

(33) Fokin V. V. *In Organic Chemistry - Breakthroughs and Perspectives*, Ding K., Dai L.-X. (Eds.) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, **2012**, 247–277

(34) Hein J. E., Krasnova L. B., Iwasaki M., Fokin V. V., *Org. Synth.*, **2011**, *88*, 238–246

(35) Berg R., Straub B. F. *Beilstein J. Org. Chem.*, **2013**, *9*, 2715–2750

(36) Ahlquist M., Fokin V. V. *Organometallics*, **2007**, *26*, 4389–4391

(37) Straub, B. F. *Chem. Commun.*, **2007**, 3868–3870

(38) Rodionov V. O., Fokin V. V., Finn M. G. *Angew. Chem.*, **2005**, *117*, 2250–2255; *Angew. Chem. Int. Ed.*, **2005**, *44*, 2210–2215

(39) Zhou D., Chu W., Dence C. S., Mach R. H., Welch M. *J. Nuclear Medicine and Biology*, **2012**, *39*, 1175–1181

(40) Kettenbach K., Schieferstein H., Ross T. L. *BioMed Research International*, volume **2014**

(41) Sirion U., Kim H. J., Lee J. H., Seo J. W., Lee B. S., Lee S. L., Oh S. J., Chi D. Y. *Tetrahedron Letters*, **2007**, *48*, 3953-3957

(42) Li Z. -B., Wu Z., Chen K., Chin F. T., Chen X. *Bioconjugate Chem.*, **2007**, *18*, 1987-1994

(43) Gill H. S., Marik J. *Nature Protocols*, **2011**, *6*, 1718-1725

(44) Devaraj N. K., Keliher E. J., Thurber G. M., Nahrendorf M., Weissleder R. *Bioconjugate Chem.*, **2009**, *20*, 397-401

(45) Lee C. -M., Jeong H. -J., Kim D. W., Sohn M. -H., Lim S. T. *Nanotechnology*, **2012**, *23*, 1-7

(46) Schieferstein H., Betzel T., Fischer C. R., Ross T. L; *EJNMMI Research*, **2013**, *3*, 1-10

(47) Maschauer S., Prante O. *Carbohydrate Research*, **2009**, *344*, 753-761

(48) Maschauer S., Einsiedel J., Haubner R., Hocke C., Ocker M., Hübner H., Kuwert T., Gmeiner P., Prante O. *Angewandte Chemie Int. Ed.*, **2010**, *49*, 976-979

(49) Schieferstein H., Ross T. L. *Eur. J. Org. Chem.*, **2014**, 3546-3550

(50) Pretze M., Mamat C. *Journal of Fluorine Chemistry*, **2013**, *150*, 25-35

(51) Kaushik B., Basudeb A., Chinmay C. *Synthesis*, **2013**, *45*, 545-555

(52) Chun J. H., Pike V. W. *Eur. J. Org. Chem.*, **2012**, 4541-4547

(53) Smith P.A.S. *The Chemistry of Open-Chain Organic Nitrogen Compounds*, **1966**, *Vol.2*, W.A. Benjamin Inc., New York, USA, 211-256

(54) *Organic Azides: Syntheses and Applications* Bräse S., Banert K. (Eds.), **2010,** John Wiley & Sons, Ltd.

(55) Bräse S., Gil C., Knepper K., Zimmermann V. *Angew. Chem. Int. Ed.*, **2005**, *44*, 5188-5240

(56) Lee J., Joeong E., Kim J. Chem. Commun., **2011**, *47*, 358-360

(57) Kumar A., Li K., Cai C. Chem. Commun., 2011, 47, 3186-3188

(58) Bridger G. J., Boehringer E. M., Wang Z., Schols D., Skerlj R. T., Bogucki D. E. *Antiviral Compounds*, US6506770 B1, **2003**

(59) Heathcock C. H., Young S. D., Hagen J. P., Pilli R., Badertscher U. *J. Org. Chem*., **1985**, *50*, 2095-2105

(60) Ferraboschi P., Grisenti P., Casati R., Fiecchi A., Santaniello E. J. Chem. Soc. Perkin Trans. I, **1987**, 1743-1748

(61) Bernady K. F., Floyd M. B., Poletto J. F., Weiss M. J. *J. Org. Chem.*, **1979**, *44*, 1438-1447

(62) Lehrfeld J. *J. Org. Chem.*, **1967**, *32*, 2544-2546

(63) Ferraboschi P., Fiecchi A., Grisenti P., Santaniello E. *J. Chem. Soc. Perkin Trans. I*, **1987**, 1749-1751

(64) Corey E. J., Niwa H., Knolle J. *J. Am. Chem. Soc.*, **1978**, *100*, 1942-1943

(65) Ding R., He Y., Wang X., Xu J., Chen Y., Feng M. and Qi C. *Molecules* **2011**, *16*, 5665-5673

(66) Amann F., Schaub C., Müller B., Schmidt R. R. *Chem. Eur. J.*, **1998**, *4*, 1106–1115

(67) Mowery B. P., Prasad V., Kenesky C. S., Angeles A. R., Taylor L. L., Feng J.-J., Chen W.-L., Lin A., Cheng F.-C., Smith A. B., Hirschmann R. *Organic Letters*, **2006**, *8*, 4397-4400

(68) Lebeau L., Pierrat P., Pons F., Zuber G. Patent n. WO2013/14073 A1, **2013**

(69) Pichika R., Eckelmann W. C., Rapoport S. I., Taha A., Kotta K. Patent n. US2013/302247 A1, **2013**

(70) Edelson B. S., Stoltz B. M., Corey E. J. *Tetrahedron Letters*, **1999**, *40*, 6729-6730

(71) Cooper G. M., *The Cell: A Molecular Approach. 2nd edition*, **2000**, Sunderland (MA): Sinauer Associates

[72] Zhang H., Lu Y., Wu S., Wei Y., Liu Q., Liu J., Jiao Q. *Journal of Molecular Catalysis B: Enzymatic*, **2016**, *124*, 38-44

[73] Bueso F., Moreno L., Cedeno M., Manzanarez K. *Journal of Biological Engineering*, **2015**, *9*, 1-7

[74] Skoronski E., de Oliveira D. C., Fernandes M., da Silva G. F., Magalhães M. D. L. B., João J. J. *Journal of Cleaner Production*, **2016**, *112*, 2553-2559

[75] Kausaite-Minkstimiene A., Ramanaviciene A., Simanaityte R., Gabrielaitis D., Glumbokaite L., Ramanavicius A. *RSC Advances*, **2015**, *5*, 105475-105483

[76] Semlitsch S., Torron S., Johansson M., Martinelle M. *Green Chem.*, **2015**, DOI: 10.1039/c5gc02597d

[77] Niu H. Shah N., Kontoravdi C. *Biochemical Engineering Journal*, **2016**, *105*, 455-472

[78] Ferraboschi P., Reza-Elahi S., Scotti L., Santaniello E. *Tetrahedron: Asymmetry*, **2000**, *11*, 2665-2668

[79] Grisenti P., Ferraboschi P., Casati S., Santaniello E. *Tetrahedron: Asymmetry*, **1993**, 4, 997-1006

[80] Chan T. R., Hilgraf R., Sharpless K. B., Fokin V. V., *Organic Letters,* **2004**, *6*, 2853-2855

[81] Borsook H., Thimann K. V. *Journal of Biological Chemistry*, **1932**

[82] Monk C. B. *Transactions of the Faraday Society*, **1951**

[83] Lai H. patent n. WO2012166810 A1, **2012**

[84] Trojer M. A., Movahedi A., Blanck H., Nydén M. *Journal of Chemistry*, **2013,** 1-23

[85] Cedzynska K., Langefelderová H., Gažo J. *Chem. zvesti*, **1981**, 35, 223-228

[86] Beck W. *Z. Naturforsch.*, **2009**, *64b*, 1221-1245

[87] Vassiliou S., Xeilari M., Yiotakis A., Grembecka J., Pawelczak M., Kafarski P., Mucha A. *Bioorg. Med. Chem.*, **2007**, *15*, 3187-3200

[88] Schwede W., Schmees N., Moeller C., Schmidt A., Fuhrmann, U. *PCT Int. Appl.*, **2009**, WO 2009071252 A1