

DESIGN AND SYNTHESIS OF CXCR4-SPECIFIC TRACERS FOR POSITRON EMISSION TOMOGRAPHY

A thesis submitted by

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in partial fulfilment of the requirements for the degree of

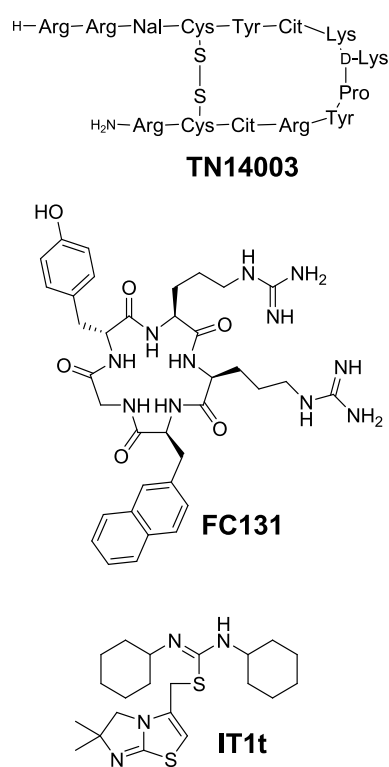
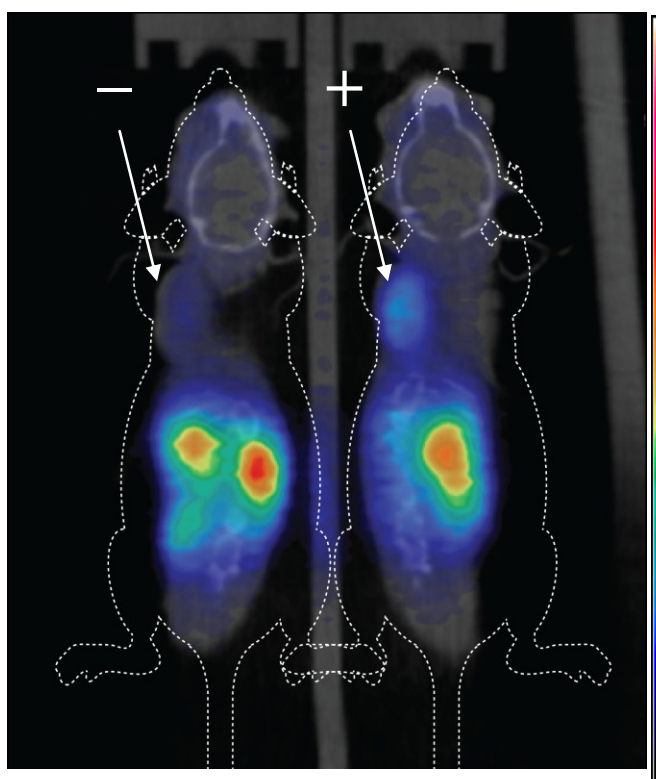
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ABSTRACT

Molecular imaging is an ideal platform for non-invasive detection and assessment of cancer. In recent years, the targeted imaging of CXCR4, a chemokine receptor that has been associated with tumour metastasis, has become an area of intensive research. CXCR4 is a GPCR whose interaction with its natural ligand SDF-1 α is essential for embryo development and haematopoiesis. Over-expression of this receptor is associated with aggressive types of cancer and potentially metastatic tumours. Several classes of CXCR4 inhibitors have been reported in the literature, including TN14003-, FC131- and IT1t-derivatives.



The aim of the research presented herein was to develop CXCR4-targeted probes for positron emission tomography. To this end, we designed and synthesised 20 new potential tracers belonging to those three classes of CXCR4 inhibitors, for labelling with fluorine-18 or gallium-68. *In vitro* and *in vivo* evaluation of these radiotracer candidates allowed for further understanding of the structure-activity relationship and pharmacokinetic properties of these types of compounds. In particular, our gallium-68-labelled tracer based on the structure of TN14003 displayed the essential features for the identification of CXCR4-expressing tumours in a clinical setting.

DECLARATION OF ORIGINALITY

I, Guillaume George, hereby confirm that I solely produced the presented thesis under the supervision of Prof. Eric O. Aboagye at the Comprehensive Cancer Imaging Centre, Department of Surgery and Oncology, Imperial College London, and Prof. Alan C. Spivey at the Department of Chemistry, Imperial College London, and that I did not use any other material than cited or known to the public domain.

Elizabeth Stevens (Imperial College London) generated all the biological data presented in this thesis (with the exception of metabolite studies). Federica Pisaneschi (Imperial College London) also contributed to the work presented herein, namely the radiosynthesis and metabolite study of [^{18}F]**35** and various deliveries of [^{18}F]**28**. The corresponding experimental procedures are described in order to offer full understanding of the corresponding results.

London, September 27th, 2013.

Guillaume George



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ABBREVIATIONS

International system of unit (SI) prefixes (p, n, μ , m, k, M, G), SI, SI-derived and SI-accepted units ($^{\circ}\text{C}$, Hz, ppm, m, $\text{\AA}$, $^{\circ}$, g, L, mol, s, min, h, eV) and combination thereof are used throughout this document, as well as three-letter amino acid codes (Xaa, Arg, Lys, etc.) and one-letter amino acid codes (X, R, K, etc.). Usual mathematical functions (such as $\ln$, $\log$) and widely-accepted chemical conventions (such as D, L, *R*, *S*, Me, Et) are not covered below.

18-crown-6 1,4,7,10,13,16-hexaoxacyclooctadecane

$[\alpha]_{\text{D}}^{20}$ specific rotation

β^{+} positron

δ chemical shift

λ wavelength

ν wavenumber

Ac acetyl

anh. anhydrous

aq aqueous

ANOVA analysis of variance

Bn benzyl

Boc *tert*-butoxycarbonyl

Bq Becquerel

br broad

BSA bovine serum albumin

c concentration

Cbz carboxybenzyl

CC₅₀ half maximal cytotoxic concentration

CD4 cluster of differentiation 4

Ci Curie

Cit citrulline

COD 1,5-cyclooctadiene

CPM count per minute

CT computerised tomography

CuAAC	copper(I)-catalysed azide-alkyne cycloaddition
CXCR4	C–X–C chemokine receptor type 4
Cy	cyclohexyl
d	doublet
DAST	diethylaminosulfur trifluoride
DBU	1,8-diazabicycloundec-7-ene
DC-RCY	decay-corrected radiochemical yield
Dde	<i>N</i> -[1-(4,4-di-methyl-2,6-dioxocyclohexylidene)ethyl
Deoxo-Fluor [®]	<i>bis</i> (2-methoxyethyl)aminosulfur trifluoride
DIPEA	ethyldiisopropylamine
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's modified Eagle's medium
DMF	<i>N,N</i> -dimethylformamide
DO3A	1,4,7- <i>tris</i> (carboxymethylaza)cyclododecane-10-azaacetyl
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
<i>D</i> _{pH 7.4}	distribution coefficient at pH 7.4 between <i>n</i> -octanol and PBS
DPPA	diphenylphosphorylazide
E1 _{CB}	conjugated unimolecular base-induced elimination
EC ₅₀	half maximal effective concentration
EDCI.HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EI	electron ionisation
EOS-RCY	end-of-synthesis radiochemical yield
equiv.	equivalent
ESI	electrospray ionisation
FBS	foetal bovine serum
FDG	2-deoxy-2-fluoro-D-glucose
FEA	2-fluoroethyl azide
Fmoc	fluorenylmethyloxycarbonyl
Fta	[1-(2-fluoroethyl)-1 <i>H</i> -1,2,3-triazol-4-yl]alanine
g	gaseous
GPCR	G protein-coupled receptor
HBTU	<i>O</i> -(benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium PF ₆
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	human immunodeficiency virus
HOBt	hydroxybenzotriazole
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectroscopy
IC ₅₀	half maximal inhibitory concentration
ID	injected dose
<i>J</i>	coupling constant
K _{2.2.2}	1,10-diaza-4,7,13,16,21,24-hexaoxabicyclo[8.8.8]hexacosane
<i>K</i> _{ML}	complex stability constant
<i>K</i> _{sp}	soluble product constant
L-DOPA	(<i>S</i>)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid
m	multiplet, medium
<i>m</i>	mass
M	molar
m.p.	melting point
MS	mass spectrometry
<i>n</i>	number of independent experiments
N	normal
<i>N</i> _A	Avogadro's number
Nal	2-naphthylalanine
NMR	nuclear magnetic resonance
NO ₂ A	1,4- <i>bis</i> (carboxymethylaza)cyclononane-7-azaacetyl
NOTA	1,4,7-triazacyclononane-1,4,7-triacetic acid
NUV ₍₆₀₎	normalised uptake value (at 60 min post-injection)
o.n.	overnight
Orn	ornithine
OSEM3D	3D ordered subset expectation maximisation
P	<i>p</i> -value
Pbf	<i>N</i> -2,2,4,6,7-pentamethyl-dihydro-benzofuran-5-sulfonyl
PBS	phosphate buffered saline (pH 7.4)
PDB	protein data bank
Pd/C	palladium on carbon
PEG	polyethyleneglycol, (–CH ₂ –CH ₂ –O–) _n
PET	positron emission tomography

pH	potential hydrogen
Prg	propargylglycine
PTFE	polytetrafluoroethylene
Py	pyridine
q	quadruplet
RCY	radiochemical yield
rel. int.	relative intensity
R _f	retardation factor
RIPA	radioimmunoprecipitation assay
ROI	region of interest
rpm	rotation per minute
RPMI	Roswell Park Memorial Institute
r.t.	room temperature
s	strong, singlet
SAR	structure-activity relationship
SD	standard deviation
SDF-1 α	stromal cell-derived factor-1 α
SelectFluor	1-(chloromethyl)-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane ditetrafluoroborate
SEM	standard error of the mean
S _N 2	bimolecular nucleophilic substitution
S _N Ar	aromatic nucleophilic substitution
SPE	solid-phase extraction
SPECT	single-photon emission computed tomography
t	time, triplet
t _{1/2}	half-life
TAC	time <i>versus</i> activity curve
TBAI	<i>tert</i> -butylammonium iodide
tBu	<i>tert</i> -butyl
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
TFA	trifluoroacetic acid, trifluoroacetate
TFE	trifluoroethanol
THF	tetrahydrofurane
TIS	triisopropylsilane
TLC	thin layer chromatography

TMEDA	tetramethylethylenediamine
Ts	tosyl
UV	ultraviolet
<i>v</i>	volume
VEGFR-2	vascular endothelial growth factor receptor 2
vs	very strong
w	weak
XtalFluor-E [®]	(diethylamino)difluorosulfonium tetrafluoroborate
XtalFluor-M [®]	difluoro(morpholino)sulfonium tetrafluoroborate
<i>z</i>	charge

Aleva, sesqui distus.

—Perus

À Papoute et Mamoute,

CHAPTER I.

INTRODUCTION

1. Cancer

Cancer is a large group of diseases characterised by unregulated cell growth. Malignant tumours invade surrounding tissues and can spread further through metastasis, *via* the lymphatic and blood vessels.

12.7 million cases of cancer were reported in 2008, and 7.6 million people died from it, making cancer the first cause of death in developed countries and the second cause in developing countries.¹ This reflects the incidence of age and lifestyle in its development. It is considered that 90–95% of the causes are related to lifestyle, from the consumption of tobacco and exposure to ionising or UV radiations, to certain infections or environmental pollutants. The remaining 5–10% are believed to be genetic factors.²

In 2000, Hanahan and Weinberg³ published a seminal article, whereby they theorise that cancers are differentiated from normal healthy cells by six hallmarks, later updated with four new hallmarks.⁴ Cancer therefore requires the acquisition of these physiological changes for malignant growth, although the reasons and the chronology for doing so can widely differ. In order to replicate uncontrollably, tumour cells need be self-sufficient in growth signals, insensitive to antigrowth signals from neighbouring cells and able to evade apoptosis. Moreover, they need to obtain an unlimited potential for replication, to foster blood vessel formation to sustain tumour growth and to acquire the ability to invade surrounding tissues and form new colonies ("metastases") in distant sites. Metastasis leads to 90% of human cancer deaths.³

The phenotype of tumour cells differs from normalcy by the up-regulation or down-regulation of certain genes or expression of proteins.³ An example of protein down-regulation can be found among the many biochemical mechanisms underlying the appearance of metastatic behaviour: for instance, cell–cell and cell–extracellular matrix adhesion can be overcome by

mutational inactivation, translational repression or proteolysis of the corresponding adherence proteins.³ Conversely, an example of protein up-regulation can be found in CXCR4.

2. C–X–C chemokine receptor type 4

2.1. Nature and structure

Discovered in 1994,⁵ C–X–C chemokine receptor type 4 (CXCR4) is a cell surface receptor of particular interest in cancer research as it is over-expressed in particularly aggressive types of cancer and/or metastatic tumours.^{6,7}

Its natural ligand, the stromal cell-derived factor-1 α (SDF-1 α) is a chemokine.^{8,9} As such, its interaction with CXCR4 provokes chemotaxis, *i.e.* guided movement of the cell in response to chemical stimulation. Briefly, the chemical signal (SDF-1 α) is transduced through the cell wall and into the cell *via* the conformational change undergone by the receptor upon binding. This flexibility of the receptor is a key characteristic of G protein-coupled receptors (GPCRs), a class of receptor to which CXCR4 belongs. This conformational change then causes an amplifying signalling cascade eventually resulting in the release of calcium ions from intracellular stores into the cytoplasm. This eventually generates chemotaxis *via* further signalling cascades.^{10,11}

Like other GPCRs, CXCR4 structurally consists of a canonical bundle of seven transmembrane α -helices disposed in circle and connected by intra- and extracellular loops. The binding site of the natural ligand is found within the helix bundle, on the extracellular side. In 2010, Stevens and co-workers resolved the crystal structure of CXCR4.¹² In particular, CXCR4 was co-crystallised with two different antagonists: CVX15 and IT1t, a hexadecapeptide and a small molecule, respectively (Figure 1) (see section I.2.3).

Moreover, GPCRs are known to form homo- and heterooligomers, which have been shown to have implications on receptor signalling.^{13,14} In particular, homodimeric structures of CXCR4 have been found in the presence and in the absence of ligand SDF-1 α ,^{15,16} therefore undoubtedly influencing structure of CXCR4's binding site and response to ligands.



Figure 1. Crystal structure of CXCR4, co-crystallised with small molecule antagonist IT1t. The receptor crystallised as homodimer.

2.2. The CXCR4 – SDF-1 α axis

CXCR4 has a wide range of roles in the healthy organism. It is involved in the migration of cells during embryonic development and plays a role in adult life, including inflammatory response and mobilisation of hematopoietic stem cells.^{17,18}

Since its discovery,⁵ over-expression of CXCR4 has been associated with various pathologies, including human immunodeficiency virus (HIV) infection,¹⁹ aggressive types of cancer and metastatic tumours.^{6,7} More precisely, malignant tumour cells respond to a chemokine gradient to home in on and infiltrate distant SDF-1 α -secreting organs, such as lymph nodes, lung, liver and bone marrow,²⁰ which are common sites of breast cancer and melanoma metastases. This was corroborated by the finding that CXCR4's level of expression is higher in metastatic than in primary tumours,^{21,22} and that changes in CXCR4 signalling significantly altered metastatic burden in animal models.²³⁻²⁷

2.3. Agonists and antagonists

Early attempts at discovering potent and clinically viable CXCR4 antagonists were mainly focused on peptides. The discovery of tachyplesin I and polyphemusin II^{28,29} and their initial

structure-activity relationship (SAR) analysis that yielded octadeca- to tetrapeptidic analogues will be discussed thoroughly in chapters II and III of this thesis (Figure 2).

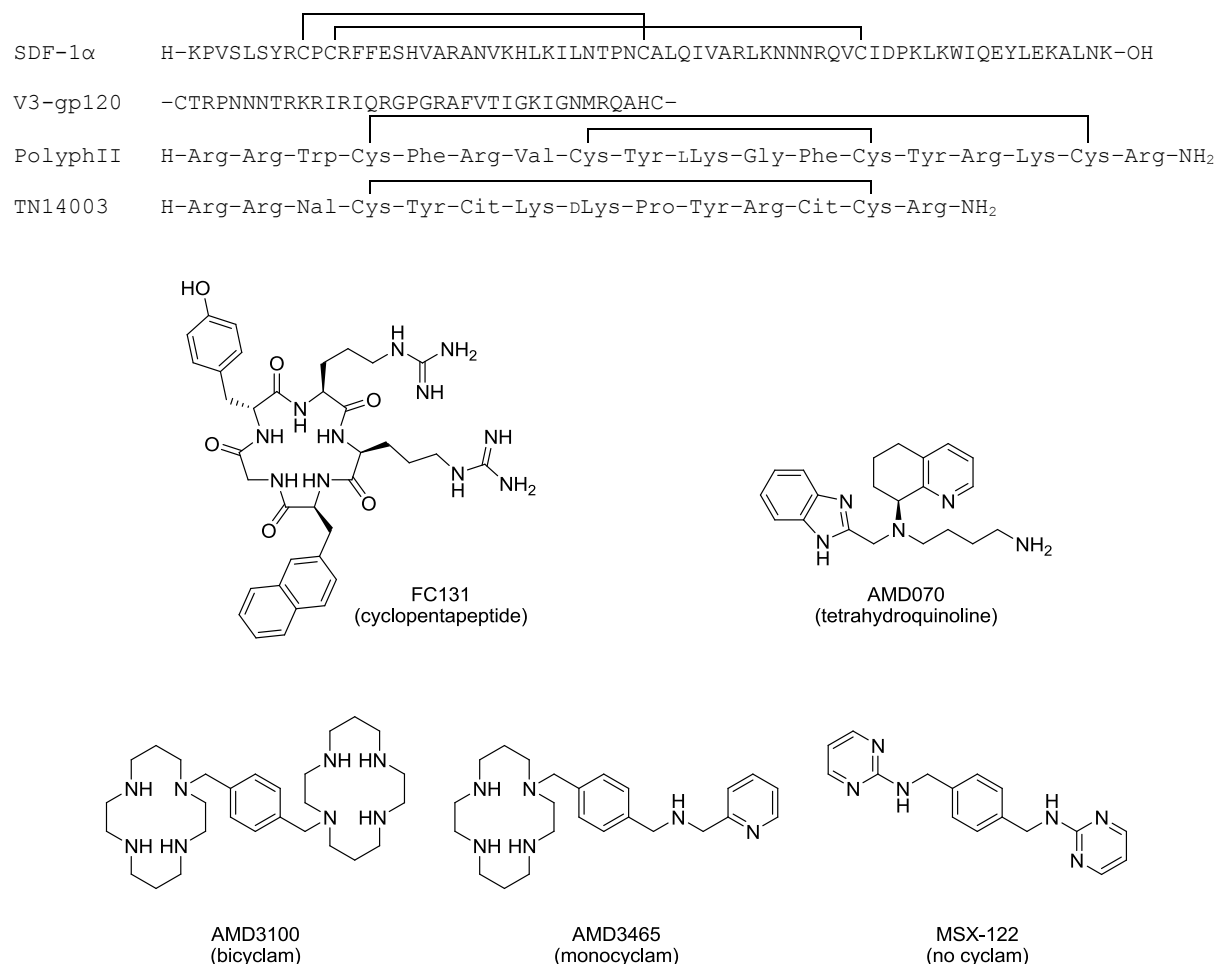


Figure 2. Selected examples of CXCR4 antagonists. Nal: 2-Naphthylalanine. Cit: Citrulline.

As outlined above, SDF-1 α is the natural ligand for CXCR4 and is therefore an agonist for this heptahelical transmembrane receptor. Gene therapy based on the over-expression of SDF-1 α is being assessed in phase I clinical trials for the stem cell-based treatment of ischemic heart diseases, for instance.³⁰

Another natural ligand of CXCR4 is the envelope glycoprotein gp120 of HIV-1, which allows entry of the virus in CD4-expressing cells, such as T-cells and macrophages,¹⁹ *via* the positively-charged 296–331 fragment ("V3 loop", or third variable region).

Antibodies are another class of biomolecules showing affinity for CXCR4.³¹⁻³³ While 12G5, 6H8 and 9E10 have been used in binding assays with CXCR4, MDX-1338 has entered phase I clinical study for acute myeloid leukaemia, non-Hodgkin lymphoma, chronic lymphoid leukaemia and multiple myeloma.³⁴

Finally, a plethora of small molecules was designed to inhibit CXCR4, a subject which was recently thoroughly reviewed by Debnath and co-workers.³⁵ These small molecules displayed a variety of chemical structures along with a strong positive charge as a common feature. Bicyclam AMD3100 was the first potent non-peptidic CXCR4 antagonist and is approved by the United States of America Food and Drug Administration for haematopoietic stem cell mobilisation (Figure 2). Removal of one (*e.g.*, monocyclam AMD3465) or two (*e.g.*, tetrahydroquinolines, *para*-xylene derivatives) of the rings afforded compounds of clinical interest; for instance, compounds AMD070 and MSX-122 are in phase I/II and phase I clinical trials, respectively.^{36,37}

3. Positron emission tomography

3.1. Principles of positron emission tomography

Positron emission tomography (PET) is based on the visualisation of γ photons resulting from the annihilation of an electron with a positron. In particular, this imaging modality is non-invasive and highly sensitive with a spatial resolution of up to ~ 2.4 mm for clinical settings.³⁸

It allows the imaging of biological processes or of the over-expression of a specific receptor or enzyme by the introduction of a positron-emitting radionuclide into a targeting molecule. After reformulation into an injectable solution and quality control, the resulting tracer (also known as probe) is injected into the patient's cardiovascular system. If well designed, the tracer accumulates in cells accordingly to the process of interest. Upon radioactive decay, the radioisotope has a certain probability to produce a positron of defined kinetic energy. Thus, fluorine-18 has a half-life of ~ 110 min and a 97% probability of emitting a positron of 640 keV (the remaining 3% corresponds to electron capture), while copper-64 has a half-life of ~ 12.7 h and a 18% probability of emitting a positron of 653 keV (the remaining 82% corresponds to β^- decay, electron capture and internal conversion). When most of the positron's kinetic energy is transferred to the environment, it undergoes annihilation with a neighbouring electron. The event produces two quasi-antiparallel γ photons of 511 keV each,

which can be detected by a ring-shaped set of γ cameras. Computational processing then allows determination of the localisation of the annihilation event.

3.2. Radionuclide production

Most positron-emitting radionuclides are produced by a particle accelerator, such as a cyclotron.³⁹ A charged particle is accelerated and collided with a target atom, which then undergoes a nuclear reaction. For instance, there are two main ways of producing fluorine-18.⁴⁰ Collision of a proton with [^{18}O]H₂O forms nucleophilic [^{18}F]fluoride by the nuclear reaction $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$. Conversely, collision of deuterons with ^{20}Ne affords electrophilic [^{18}F]F₂ *via* $^{20}\text{Ne}(\text{d},\alpha)^{18}\text{F}$. The production of most PET-relevant isotopes requires an on-site cyclotron because of their short half-life (20.4 min for ^{11}C , 9.97 min for ^{13}N and 2.04 min for ^{15}O). The price of such devices therefore limits their use in clinical settings. On the other hand, it is possible to produce other radionuclides (such as ^{18}F or ^{64}Cu) off-site and transfer them to the hospital for use.

An alternative to cyclotrons are radioisotope generators, devices that are used to separate and extract a radioisotope from its parent radioisotope. In particular, when a parent isotope of long half-life is adsorbed onto an inorganic or polymeric matrix and decays into the radioisotope of interest of shorter half-life, this can be eluted off the matrix to be incorporated into a molecule of interest. This technique is particularly attractive as it allows production of the relatively long-lived parent radioisotope on one site and its transfer for the use of the daughter radioisotope on another site. An example of such generator is the $^{68}\text{Ge}/^{68}\text{Ga}$ generator.⁴¹ Germanium-68 has a half-life of 271 days, and is produced in many different ways, including $^{69}\text{Ga}(\text{p},2\text{n})^{68}\text{Ge}$ and $^{66}\text{Zn}(\alpha,2\text{n})^{68}\text{Ge}$. Within a few hours, the amount of gallium-68 contained in the matrix reaches steady-state, meaning it is possible to elute the generator several times a day. In modern generators, this is performed with aqueous acid (0.1–1.0 M HCl_(aq)) in less than 2 min. The resulting acidic eluate can be used directly or concentrated by means of a cation exchange cartridge. The whole elution process can be automated; the generator and the automaton can be installed in a standard-size shielded fumehood and used for up to 2 years.

3.3. Tomography

Once generated, the radioisotope is then reacted with a precursor to afford, after purification and reformulation into an injectable solution, the desired tracer (see section I.5). The tracer is injected to the patient and accumulates in targeted organs or cells. As explained earlier, upon

decay of the radioactive probe, a pair of quasi-antiparallel γ rays are produced and detected by the scanner.

Within the detector, a scintillator absorbs the γ photon and re-emits the absorbed energy in the form of light. Photomultipliers effectively convert this electromagnetic signal into an electronic signal, which is processed by a computer. An array of these in-series scintillator–photomultiplier couples are arranged in a ring shape in order to picture ("graphein") sections ("tomos") of the scanned body. Put together, these sections form a three-dimensional representation of the localisation of the radiotracer.

3.4. PET tracer requirements

The following discourse is intended to introduce some important facets relative to PET tracer chemistry and to offer the reader an appreciation of some of the specificity of many techniques for PET imaging based on my personal experience.

3.4.1. Radiolabelling

PET imaging requires a certain amount of radioactivity to be injected in the subject of the experiment. Typically, a human scan necessitates 370 MBq (10 mCi), while 3.7 MBq (100 μ Ci) is enough for mouse *in vivo* study. Two different measures of the radiochemical yield (RCY) can be reported: the aptly named decay-corrected radiochemical yield (DC-RCY) and end-of-synthesis radiochemical yield (EOS-RCY).⁴² Whereas DC-RCY is related to the completion of one particular radiochemical transformation from the chemical point of view and accounts for the loss of activity as a function of time due to radiodecay, EOS-RCY is orientated towards the delivery in itself, taking into account the influence of the duration of the labelling on the synthesised amount of activity. In order to maximise the amount of activity delivered, the radiolabelling procedure can be optimised by either improving the DC-RCY or increasing the reaction rate. This is achievable by screening reaction conditions (such as quantity of precursor, concentration of the reaction mixture, reaction temperature, reaction duration, pH, solvents and reagents) along with careful monitoring of the reaction. In general, improvement of the RCY can be achieved by reducing the number or complexity of steps, thus limiting the total duration of the radiolabelling procedure and the losses of material due to manipulation. Finally, inefficient syntheses might require one to start with higher amounts of starting activity, which increases the dose received by the radiochemist and produces further issues (*e.g.*, radiolysis, necessity for automation).

After purification, generally by high pressure liquid chromatography (HPLC), the tracer needs to be reformulated into an injectable solution. Indeed, common HPLC organic solvents (*e.g.*, MeCN, MeOH) or additives (*e.g.* trifluoroacetic acid, TFA) are toxic. Reformulation is normally performed by trapping the tracer onto a solid-phase extraction (SPE) cartridge, washing toxic chemicals off with water or phosphate buffer saline (PBS) and eluting the tracer off the cartridge with EtOH. Partially irreversible trapping can be observed, requiring optimisation of the reformulation step. Moreover, the content of EtOH injectable is limited to 10% (v/v) in PBS.

Before administration, the quality of the delivered material is controlled; this usually takes the form of analytical HPLC or radio-TLC for animal studies, while deliveries for human imaging undergo further analysis (such as gas chromatography, mass detection, pH value, osmolarity, sterility).⁴³ From this, two physical quantities can be determined. First, the radiochemical purity is determined as the ratio of the area under the tracer's peak to the total area in the radiochromatogram. Second, the UV-chromatogram allows determination of the amount of co-eluting tracer-like species in the delivered material by means of a calibration curve. From this, calculation of the apparent specific activity as the ratio of radioactivity per amount of material (typically in GBq.μmol⁻¹) is possible.⁴² This last physical quantity plays an important role in receptor imaging, as will be discussed in greater detail later (see section III.2.3). Briefly, a sample of low specific activity contains a large proportion of non-radioactive tracer-like molecules, which can saturate the receptors and thus reduce the uptake of the tracer. A minimal specific activity is however difficult to agree on as it greatly depends on receptor expression and receptor affinity of the tracer.

An aqueous solution of the tracer is used to confirm the stability of the tracer over clinically-relevant length of time.

Finally, reproducibility is an important feature for radiosynthesis, as setting up deliveries requires scheduling numerous other aspects such as cell cultures, acquisition of mice, mice xenografting and room and equipment booking. The reproducibility can be assessed by the standard deviation of the EOS-RCY for instance. Automation can improve reproducibility and is the only way tracers can be produced in clinical settings, although the development of such protocols can be time-consuming.

3.4.2. Pharmacokinetics

Distribution

The biodistribution of the tracer is important when designing a PET probe, as high accumulation in the tumour and low accumulation in other organs is desired. Various factors influence distribution of the radiotracer, including metabolism and excretion as discussed later in this thesis (see chapters II and III).

The tracer needs to exhibit binding affinity for the receptor it targets. This affinity should be potent enough for the tracer to bind quasi-irreversibly. Moreover, the tracer must have specificity for the targeted receptor. Indeed, members of the same receptor family might present structural similarities but different function or biodistribution. Therefore, a tracer of poor specificity could target other cells, organs or biological processes than initially intended, leading to false positives or increased background.

Another cause of reduced signal-to-noise ratio is non-specific binding. This is, for instance, apparent for lipophilic tracers that bind non-specifically into cell membranes, therefore diminishing contrast. Although this process is sometimes desirable for drugs, as it allows the molecules to evade metabolism or excretion and to be slowly released into the blood stream, the half-life of radiotracers is generally too short for this process to be advantageous. Tracers synthesised with isotopes of longer half-lives (such as copper-64, with a half-life of 12.7 h) can be used in order to circumvent such issues. In these approaches, the tracer is administered to the patient and allowed to incubate for several hours. Meanwhile, the tracer accumulates in the tumour while non-specific binding is washed off, thus improving contrast. This strategy however increases the dose to and the inconvenience for the patient. The pre-targeting strategy,⁴⁴ whereby the patient is injected with a non-radioactive targeting molecule that bears a functional group, was devised to circumvent this issue. After incubation, a molecule that is able to react bioorthogonally with the functional group is labelled with a short-lived positron-emitting isotope and the resulting radioactive molecule is injected to the patient. This technique makes it possible to benefit from the increase in contrast concomitant with long incubation time without the inconvenience and dose associated to the injection of a long-lived radiotracer.

Metabolism

Metabolism is a set of enzyme-catalysed chemical transformations that are necessary for maintaining life. In particular, xenobiotic metabolism is aimed at transforming chemical entities ("parent" compounds) that are foreign to the normal biochemistry of the organism, such as drugs or radiotracers, into metabolites of improved excretability or of potentially lower toxicity. Although every biological tissue has some ability to metabolise xenobiotics, the liver is the main contributor to metabolism. Other main sites for metabolism are the kidneys, lung and plasma.⁴⁵

The transformation of a tracer by the organism is undesirable for the duration of the imaging protocol, as maximal blood concentration of the tracer is sought. Moreover, because of their likely lower affinity for the targeted receptor, radioactive metabolites increase background activity. Nonetheless, in particular for longer-lived isotopes (such as copper-64), metabolism can be beneficial to some extent as it assists excretion of the radioactivity *via* the urine, thus reducing the dose received by the patient. A balanced metabolism rate is therefore optimal.

Excretion

The tracer and its metabolites are then excreted by the organism, mainly by the urine (hydrophilic metabolites) or by the hepatobiliary route (lipophilic metabolites). Ideally, tracer's metabolites are rapidly excreted by the urine in order to decrease the background activity and therefore increase contrast; on the other hand, the tracer itself should not be excreted in order to maximise its blood circulation and tumour uptake.

A particular illustration of this is the use of antibodies as tracers. Their low clearance results in high background, even a week after injection. This has been circumvented by engineering antibody mimetics⁴⁶ and antigen-binding fragments,^{47,48} owing to their much faster clearance due to their smaller size.

4. Biological evaluation

The following discussion is designed to introduce the biological experiments used in the work presented in this thesis, and is not intended to provide an exhaustive overview of all existing approaches for the biological assessment of radiotracers.

4.1. Binding assay

Binding assays were performed in order to assess the affinity of the tracers for CXCR4.⁴⁹ These assays were run *in cellulo* by competition with commercially available radiolabelled [¹²⁵I]SDF-1 α . Typically, CXCR4-expressing cells were incubated with a mixture of the assessed compound at a given concentration and [¹²⁵I]SDF-1 α at a concentration lower than its half maximal effective concentration (EC₅₀). The amount of bound radioactivity was quantified using a scintillation counter.

It can be argued that cell-based assays suffer from poor reproducibility as compared to cell-free assays, owing to the numerous known and unknown factors influencing living cells, and in this particular case internalisation of the activated CXCR4.¹¹ Assays precluding this issue exist, such as the one used by Thoma and co-workers:⁵⁰ a scintillation proximity assay with partially purified CXCR4-bearing membrane preparations, thus avoiding intracellular signalling processes. However, as tracers are for *in vivo* use, it is our belief that the use of whole cells for these assays is more biologically relevant.

4.2. Migration assay

In parallel, the ability of our compounds to inhibit chemotaxis was assessed. In this assay, a suspension of human leukemic T-cell Jurkat, pre-treated with a given concentration of the assessed compound, is placed in the upper well of a transwell plate, the lower well containing a solution of SDF-1 α . After incubation, the amount of cells in the lower well was counted.

Cell migration is a downstream effect of CXCR4 activation, and as such, the prospect of monitoring our tracer candidates' chemotaxis inhibitory power to assess their tracer-like properties was discussed. In the case of the observation of chemotaxis inhibition, the antagonistic compound can be considered to show desirable properties, as the observed inhibition requires tracer binding to the receptor. Conversely, the lack of chemotaxis inhibition does not necessarily mean that the assessed molecule has no affinity for the receptor, as many other explanations are possible: for instance, the molecule could bind to an allosteric site on CXCR4 or it could be an agonist itself. The results of this assay have therefore to be considered with caution.

4.3. Cellular uptake assay

Cellular uptake assay is a simple *in vitro* experiment to allow assessment of the preferential binding of the radiolabelled tracer for CXCR4-expressing rather than for non-CXCR4-expressing cell lines, whereby the cells are incubated with the tracer and the bound activity is counted. The ratio of bound activity between the two cell lines is a measure of the tracer's potency.

Similarly, to assess whether the observed uptake is specific, blocking studies can be carried out. To this end, cells are pre-incubated with the tracer's non-radioactive isotopologue prior to addition of the tracer. Should the tracer be specific to CXCR4, this should lead to the observation of a reduced uptake compared to non-blocked experiments.

4.4. Imaging

In vivo tomography allows semi-quantitative visualisation of the tracer's biodistribution, and is an experiment whereby mice xenografted with CXCR4- or non-CXCR4-expressing tumours are injected with the tracer. The mice then undergo a bimodal computerised tomography (CT) / PET scan. Here again, a blocking study can be performed, in our case by co-injection of a given amount of the tracer's non-radioactive counterpart. The PET imaging data, expressed in percentage of the injected dose per unit of volume (typically, in % ID/mL), can be used to generate various figures/images.

Time *versus* activity curves are obtained by plotting the averaged normalised uptake of the tumours *versus* post-injection time, allowing the kinetics of tumour penetration to be assessed, the reversibility of the binding and the *in vivo* difference of uptake between CXCR4-positive, CXCR4-low and blocked CXCR4-positive tumours. In particular, the normalised uptake value at 60 min (NUV₆₀) is often reported.

Tumour-to-muscle ratios for CXCR4-positive, CXCR4-low and blocked CXCR4-positive tumours at a given time post-injection (generally, 60 min for fluorine-18 and gallium-68) is also calculated as a measure of the signal-to-noise ratio.

Finally, inspection of the scan frames allows hypotheses about the tracer's pharmacokinetic properties to be formulated.

4.5. Biodistribution

Imaging is complemented by biodistribution experiments, for which mice are sacrificed immediately at the end of the scan, tissues harvested and their activity counted, expressed in percentage of the injected dose per unit of weight of the tissue (typically, in % ID/g). Compared to CT/PET imaging, this method is quantitative and more precise. Healthy mice can be used for dynamic (or time-course) biodistribution, however these experiments are generally limited to a few time-points for temporal, financial and ethical reasons.

4.6. Metabolite study

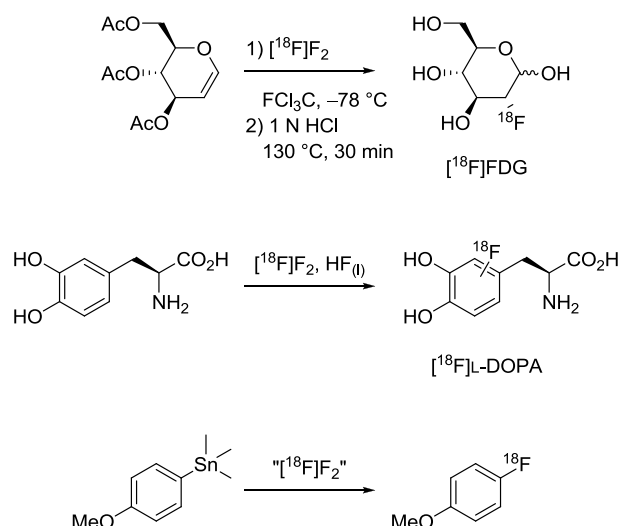
Dynamic biodistribution is normally coupled with metabolite analysis, as both experiments can be performed on the same mice. Observation in CT/PET imaging of radioactivity in the liver, the gastrointestinal tracts or the urine signifies potential metabolism and/or excretion. These, along with plasma stability, can be measured by processing various tissues for HPLC analysis. Comparison of the radiotracers of tissues' extracts with that of the parent compound allows the extent of metabolism to be quantified.

5. Radiolabelling

5.1. Electrophilic fluorine-18 chemistry

As outlined earlier, electrophilic fluorine-18 is generally produced by $^{20}\text{Ne}(\text{d},\alpha)^{18}\text{F}$ nuclear reaction, using 0.1–2.0% $^{19}\text{F}\text{F}_{2(\text{g})}$ to reduce adsorption of electrophilic fluorine-18 to the walls of the reactor. This method produces "carrier-added" radioactive fluorine gas, of low specific activity ($< 1 \text{ GBq}\cdot\mu\text{mol}^{-1}$).⁵¹

However, owing to the intrinsically high reactivity of fluoronium ions, electrophilic radiofluorination remains an area of active research. The first synthesis of 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) was performed by fluorination of the corresponding glucal with molecular fluorine-18 in 8% yield (Scheme 1).⁴⁰ Initially designed to map glucose metabolism in living human brains, ^{18}F FDG is today also used for cancer imaging and represents the most common PET tracer in clinical settings.

Scheme 1. Selected electrophilic radiofluorination reactions with $[^{18}\text{F}]\text{F}_2$.^{40,42,52}

Molecular radioactive fluorine has also been used for electrophilic aromatic substitution (*e.g.*, for the synthesis of fluorinated congeners of phenylalanine, tyrosine and L-DOPA)⁵² and various regioselective fluorodemetalation reactions with stannanes, silanes, germanes and organomercury reagents, but also organolithium and Grignard reagents.⁴²

Finally, a wide range of other electrophilic radiofluorinating agents was designed in order to temper the high reactivity of molecular fluorine and achieve more selective radiofluorination reactions. Thus, $[^{18}\text{F}]\text{XeF}_2$,⁵³ $[^{18}\text{F}]\text{AcOF}$,⁵⁴ $[^{18}\text{F}]\text{FCIO}_3$,⁵⁵ *N*- $[^{18}\text{F}]\text{fluorobenzenesulfonimide}$ ⁵⁶ and $[^{18}\text{F}]\text{SelectFluor}$ ⁵⁷ were reported in the literature for radiofluorination of a variety of substrates.

5.2. Nucleophilic fluorine-18 chemistry

Nucleophilic fluorine-18 is generally produced by $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ nuclear reaction, carried out on enriched $[^{18}\text{O}]\text{H}_2\text{O}$, therefore producing $[^{18}\text{F}]\text{fluoride}$ in aqueous solution. Owing to its size and charge, fluoride should theoretically be very nucleophilic. However, hydrogen bonding with water quenches this nucleophilicity dramatically. Therefore, the use of nucleophilic fluorine-18 entails a preliminary drying step. This is normally carried out by azeotropic distillation with anhydrous acetonitrile, at $80\text{--}120^\circ\text{C}$, under a flow of dry inert gas. More exotic strategies such as electrochemical drying have also been reported in the literature.⁵⁸

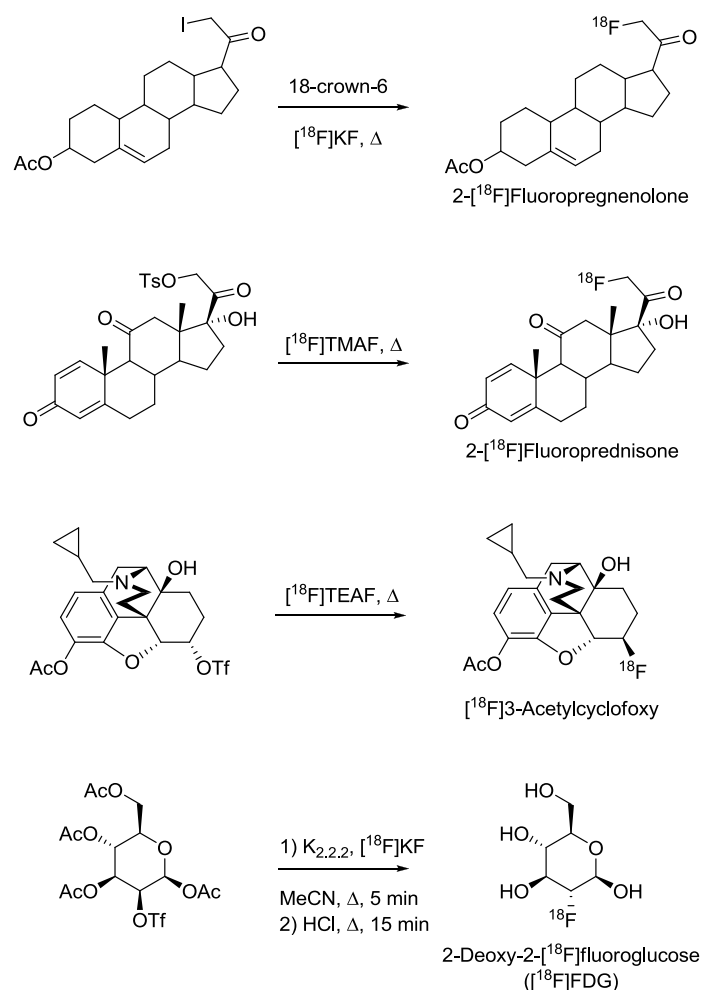
The nucleophilicity of [^{18}F]fluoride can be further improved by choosing a counter-ion that binds weakly to the fluoride ion. For instance, a system composed of K_2CO_3 or KHCO_3 along with a cryptand such as 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) or $\text{K}_{2.2.2}$ (1,10-diaza-4,7,13,16,21,24-hexaoxabicyclo[8.8.8]hexacosane) is common in radiochemistry.⁵⁹ Cs^+ and tetraalkylammonium have also been used as substitutes for the K^+ -cryptand complex.⁶⁰ Alternatively, "flexible" fluoride, or in other words the limited solvation of fluoride coordinated with bulky alcohols, has been reported in the literature with the use of solvents such as $t\text{BuOH}$.^{60,61}

The resulting nucleophilic dry mixture can then be used to perform radiofluorination. Two major types of reactions can be used to introduce [^{18}F]fluoride into an organic molecule, namely aliphatic and aromatic nucleophilic substitutions.

5.2.1. Aliphatic nucleophilic substitution

[^{18}F]Fluoride can be used to displace a leaving group in the precursor in an $\text{S}_{\text{N}}2$ fashion. The basicity of fluoride can however lead to elimination by-products.⁴² On top of this, other nucleophiles present in the reaction mixture (*e.g.*, traces of water, contaminants) compete with fluoride and are another major source of by-products.⁴² Various leaving groups have been used in this context (Scheme 2).

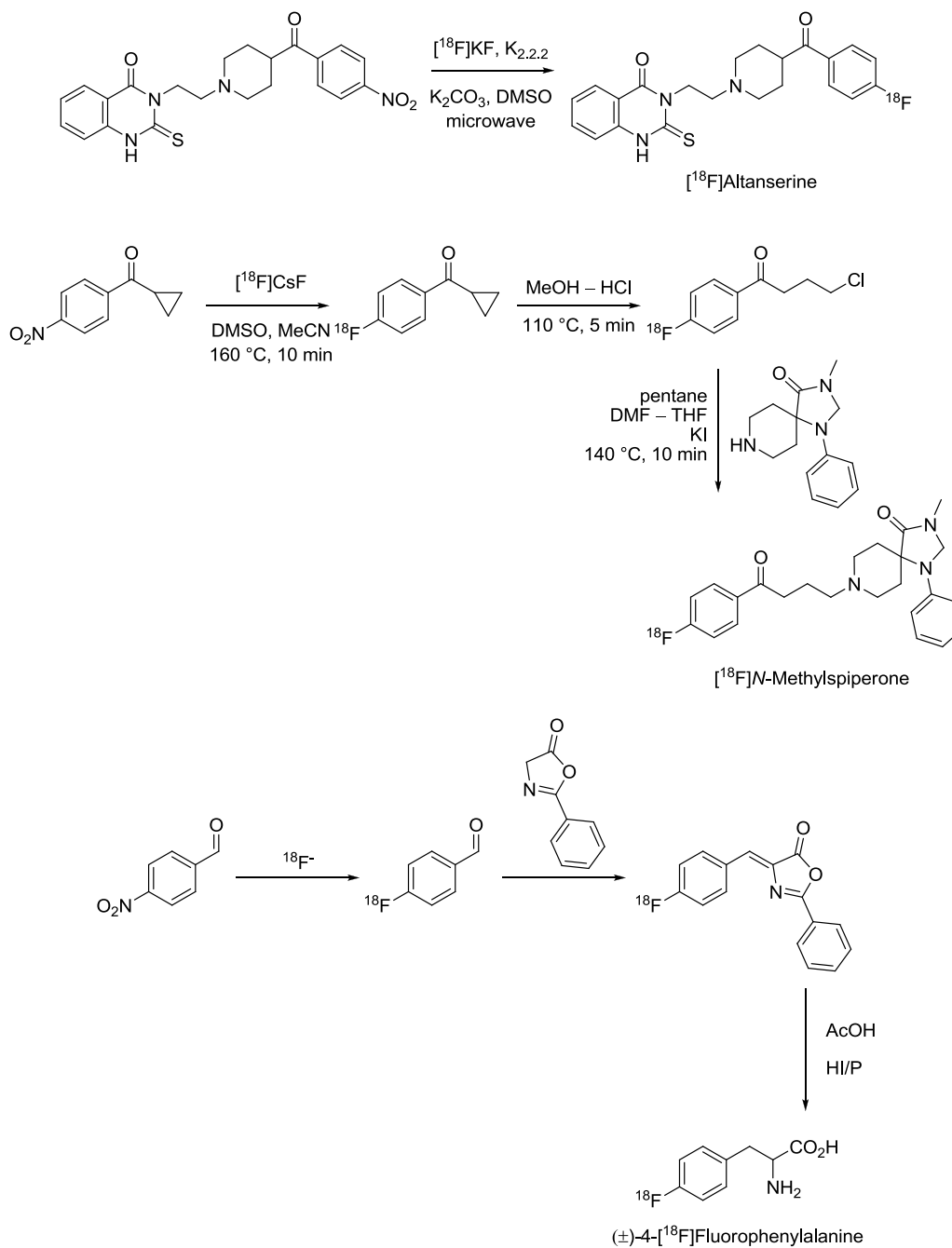
Substitution of iodide by [^{18}F]fluoride α to a carbonyl has been used for the formation of steroid hormone 2-[^{18}F]fluoropregnenolone,⁶² although in moderate DC-RCY (20%). Similarly, displacement of a tosylate leaving group to afford corticosteroid 2-[^{18}F]fluoroprednisone⁶³ was achieved in low DC-RCY (2–8%), owing to a predominant internal rearrangement reaction caused by the basicity of fluoride. The more reactive triflate leaving group can also be used as exemplified by the radiosynthesis of [^{18}F]3-acetylcyclofoxy,⁶⁴ which occurred in 35% DC-RCY. These three examples illustrate the difficulty of using $\text{S}_{\text{N}}2$ reactions to label complex molecules, due to the possible side reactions and rather drastic reaction conditions, usually involving high temperatures, anhydrous conditions and the presence of a base. However, should the tracer be of great clinical importance, dedicated efforts to circumvent these issues can be justified. For instance, the modern approach for the synthesis of the PET gold standard [^{18}F]FDG involves the [^{18}F]fluoride-for-triflate substitution in the corresponding precursor, affording the tracer in ~50% RCY.⁵⁹



Scheme 2. Selected examples of radiolabelling strategies involving the displacement of a leaving group by $[^{18}\text{F}]$ fluoride.^{59,62-64} TMAF: tetramethylammonium fluoride. TEAF: tetraethylammonium fluoride.

5.2.2. Aromatic nucleophilic substitution

The $\text{S}_{\text{N}}\text{Ar}$ mechanism allows for the substitution of an electronegative leaving group by a nucleophile, provided the aromatic ring is sufficiently electron-poor. Numerous leaving groups are possible, such as $-\text{F}$ or $-\text{NO}_2$. Although tetramethylammonium leaving groups might be preferred as their labelling can be performed at milder temperatures and charge-based isolation is possible, a competing $\text{S}_{\text{N}}2$ reaction producing $[^{18}\text{F}]$ fluoromethane is normally observed.⁶⁵ Provided the tracer conveniently features the necessary qualities, radiosynthesis can be performed in one step, as exemplified by the synthesis of $[^{18}\text{F}]$ altanserine (Scheme 3).⁶⁶

Scheme 3. Selected radiosyntheses involving aromatic nucleophilic substitutions.⁶⁶⁻⁶⁸

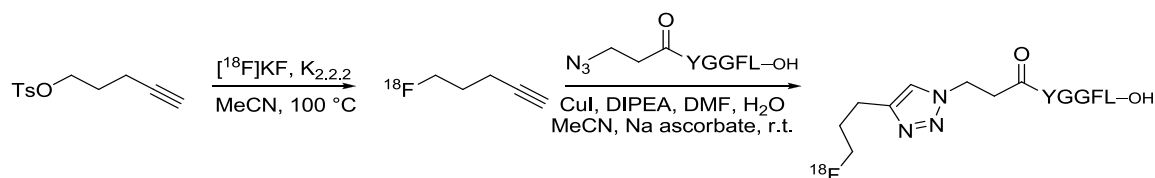
On the other hand, tracers that do not fit these requirements (either because of structural complexity or because of a lack of activation of the aromatic ring) need multistep radiosynthetic pathways, such as $[^{18}\text{F}]\text{N-methylspiperone}$ ⁶⁷ and $4\text{-}[^{18}\text{F}]\text{fluorophenylalanine}$.⁶⁸

5.2.3. Prosthetic groups

As outlined earlier, the radiosynthetic obstacles concomitant with the use of [^{18}F]fluoride for direct labelling of a precursor are manifold. The variety of reactions available is limited, and they usually require forcing conditions. This is commonly circumvented by the use of prosthetic group approaches, whereby [^{18}F]fluoride is incorporated into an organic molecule, generally in an $\text{S}_{\text{N}}2$ or an $\text{S}_{\text{N}}\text{Ar}$ manner, which can then react with a variety of precursors. The possibilities for prosthetic groups therefore mirror the wealth of organic reactions that allow coupling of two (or more) organic species. Some of these approaches are outlined below.

Aldehyde-containing prosthetic groups can be utilised to label hydroxylamines (by oxime formation), hydrazines (by hydrazone formation) or amines (by reductive amination). 4-[^{18}F]Fluorobenzaldehyde can be synthesised by aromatic nucleophilic substitution with the corresponding tetraalkylammonium species.^{69,70} The hemiacetal moiety of [^{18}F]FDG, the synthesis of which can be fully automatised,⁷¹ can react likewise. For instance, the cyclic RGD peptide, a potent inhibitor of integrins, has been labelled with (2-(2-(2-(2-[^{18}F]fluoroethoxy)ethoxy)ethoxy)ethoxy)acetaldehyde, 4-(3-[^{18}F]fluoropropoxy)benzaldehyde, 4-[^{18}F]fluorobenzaldehyde and [^{18}F]FDG.^{72,73}

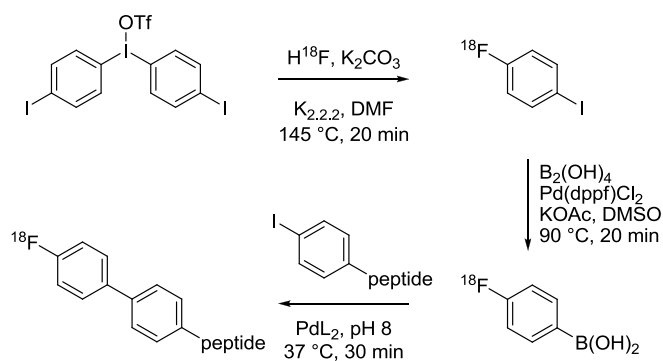
Radiolabelling can be achieved by "click" chemistry (henceforth the quotation marks will be omitted for clarity).⁷⁴ [^{18}F]Fluoroalkynes were reacted with azide-functionalised peptides in 10 min with good to excellent DC-RCY (54–99%) and radiochemical purity (81–99%) (Scheme 4).⁷⁵ Conversely, [^{18}F]fluoroalkyl azides were cyclised to alkyne-containing precursors.⁷⁶



Scheme 4. Radiolabelling by click chemistry.⁷⁵

Similarly, the thiol–maleimide reactive pair was used for radiochemistry purposes,^{69,77} for which complete conversions could be obtained even at low substrate concentrations (10 $\mu\text{g.mL}^{-1}$) in 5 min at room temperature.

Finally, examples of palladium-catalysed reactions have also been reported.⁷⁸⁻⁸⁰ Recently, a method for the labelling of 4-iodophenylalanine-containing proteins with 4- ^{18}F fluorophenylboronic acid by Suzuki-Miyaura palladium-catalysed cross-coupling was documented (Scheme 5).⁸¹ Although the method is still at its infancy (83–87% DC-RCY with small molecules, 33–48% DC-RCY with peptides and 2–5% DC-RCY with a protein), the suitability of 4-iodophenylalanine-containing proteins for protein expression⁸² vindicates encouraging perspectives for this technology.



Scheme 5. Radiolabelling by Suzuki-Miyaura palladium-catalysed cross-coupling.⁸¹ dppf: 1,1'-bis(diphenylphosphino)ferrocene.

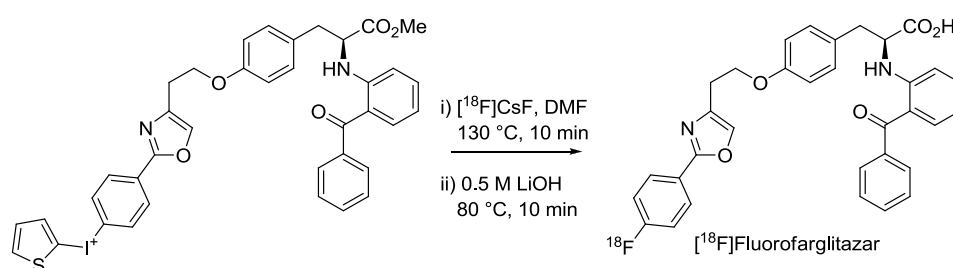
5.2.4. Formal oxidation as a means for radiofluorination

Overall, whereas the high reactivity of electrophilic fluorine-18 is tempered by concomitant low specific activities, the high specific activities obtainable by the production of nucleophilic fluorine-18 come with its lower reactivity. Efforts to combine the benefits of both strategies have led to formal oxidation approaches, whereby nucleophilic fluorine-18 is converted to " $^{18}\text{F}^{+}$ ".

In 1997, Bergmann and Solin⁵¹ converted $^{18}\text{F}^{-}$ into $^{18}\text{F}_2$ in two steps. Synthesis of $^{18}\text{F}^{-}$ from iodomethane and purification by gas chromatography was followed by atomisation by electrical discharge with a controlled amount of F_2 to afford $^{18}\text{F}_2$ by isotope

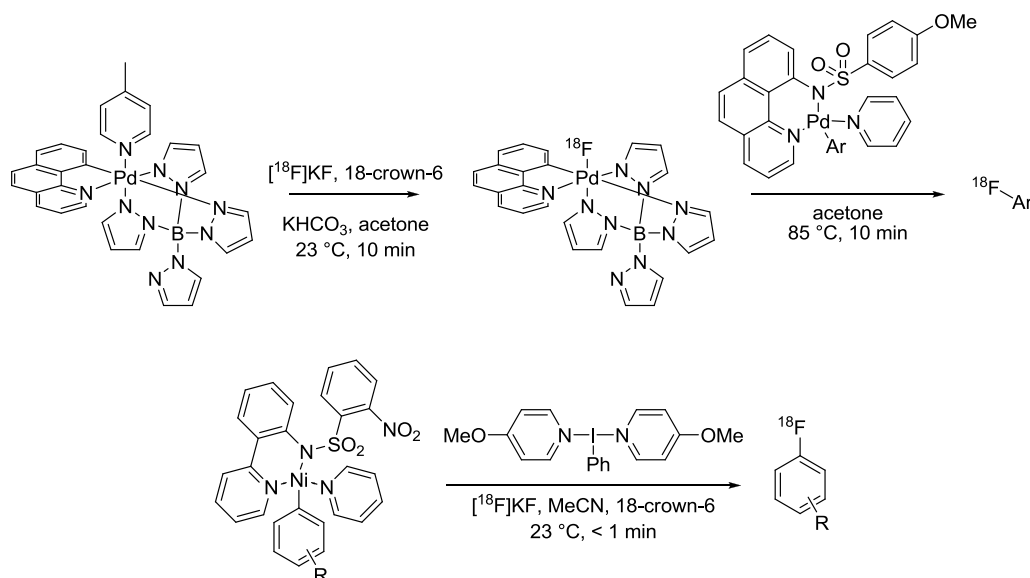
exchange. This method allowed to obtain specific activities greater than $50 \text{ GBq} \cdot \mu\text{mol}^{-1}$ in 20% RCY, starting from 37 GBq of aqueous $[^{18}\text{F}]\text{fluoride}$.

Diaryliodonium salts have been shown to label a wide range of aromatic substrates, using $[^{18}\text{F}]\text{fluoride}$ with high specific activities ($>75 \text{ GBq} \cdot \mu\text{mol}^{-1}$).⁸³⁻⁸⁵ Electron-rich rings could be labelled, provided the other aromatic ring is comparatively more electron rich. For instance, Katzenellenbogen *et al.*⁸⁶ performed radiofluorination of a derivative of farglitazar showing affinity for $\text{PPAR}\gamma$, a receptor expressed in a variety of tumour cells, in 35% DC-RCY and $37 \text{ GBq} \cdot \mu\text{mol}^{-1}$, starting from 3.7 GBq of aqueous $[^{18}\text{F}]\text{fluoride}$ (Scheme 6).



Scheme 6. Katzenellenbogen's diaryliodonium species as a means for radiofluorination.⁸⁶

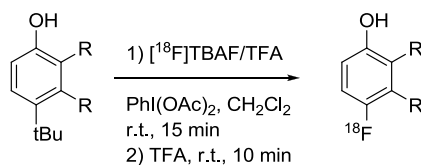
While the methods described above need harsh conditions or long procedures, Ritter and co-workers^{87,88} demonstrated the possibility to formally oxidize $[^{18}\text{F}]\text{fluoride}$ to " $^{18}\text{F}^+$ " by chelation to a palladium or a nickel complex (Scheme 7).



Scheme 7. Ritter's palladium- and nickel-based radiolabelling with aqueous [^{18}F]fluoride.^{87,88}

The reaction could be performed with aqueous fluoride, and was complete within a minute.

Alternatively, Gouverneur *et al.* developed a metal-free oxidative fluorination method for phenols, whereby the phenol is oxidised by an iodonium species and re-aromatises by loss of a *tert*-butyl (Scheme 8).⁸⁹



Scheme 8. Gouverneur's metal-free oxidative fluorination of phenols.⁸⁹

This technique is particularly attractive as it allows for the radiolabelling of electron-rich substrates in less than 30 min at room temperature.

5.3. Radiometal chemistry

5.3.1. Metal chelation

Radiometals can be used to label molecules of interest. The labelling is normally carried out by chelation to a multidentate molecule that present a high dissociation constant, such as ethylenediaminetetraacetic acid (EDTA), or its cyclised congeners 1,4,7-triazacyclononane-

1,4,7-triacetic acid (NOTA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (Figure 3).

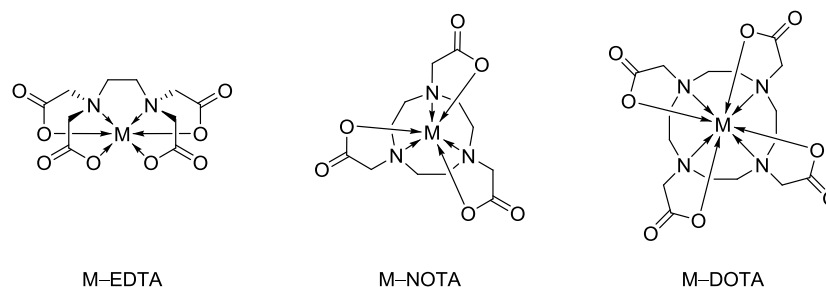


Figure 3. Structures of EDTA, NOTA and DOTA.

Many more chelating moieties have been used for radiolabelling with metals, which have been very comprehensively reviewed by Wadas and co-workers.⁹⁰

The rapidity and cleanliness of radiometal chelation are particularly interesting for tracer production. Chelation is carried out in aqueous solution and because of the high affinity of the metal cation for the chelating motif, the reaction readily happens in dilute samples, thus allowing for high specific activities. Moreover, no by-product is normally observed, allowing for fast purification by mere solid-phase extraction. However, this labelling approach requires a sizeable multidentate chelating moiety in the structure of the precursor.⁹⁰ Provided that the site for derivatisation is well-chosen, peptides, because of their larger size, can easily accommodate such modifications without major loss of binding affinity for its target.⁹¹

5.3.2. *Theranostics*

Patient stratification, made possible by targeted molecular imaging and the resulting understanding of the individualised cellular phenotype of the disease, allows for personalised medicine.⁹² In the case of radiometal PET imaging agents, the positron-emitting radionuclide could be substituted by radionuclides suitable for radiotherapy, such as ⁶⁷Cu, ⁹⁰Y, ¹⁷⁷Lu and ²¹³Bi.⁹³ This would enable theranostic dual-functionality, in a similar manner to that used for neuroendocrine tumours.⁹⁴

6. CXCR4-imaging

Some of the molecules that show affinity for CXCR4 were derivatised in order to allow for radiolabelling with fluorine-18, copper-64 and gallium-68 and some of the resulting radiotracers have been assessed in tumour models (Figure 4 and Table 1).

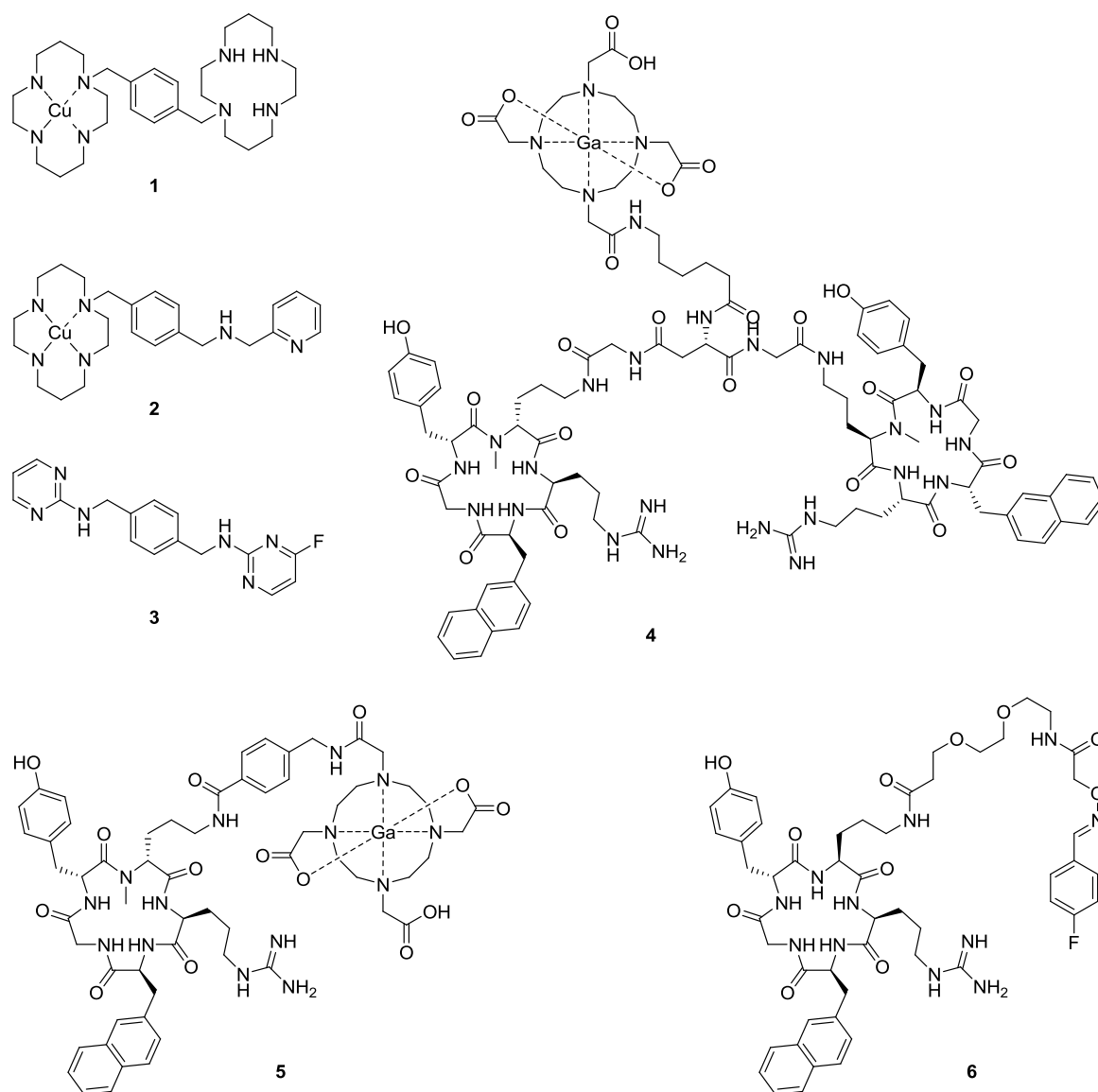


Figure 4. Compounds radiosynthesised as CXCR4-targeting PET imaging agents.

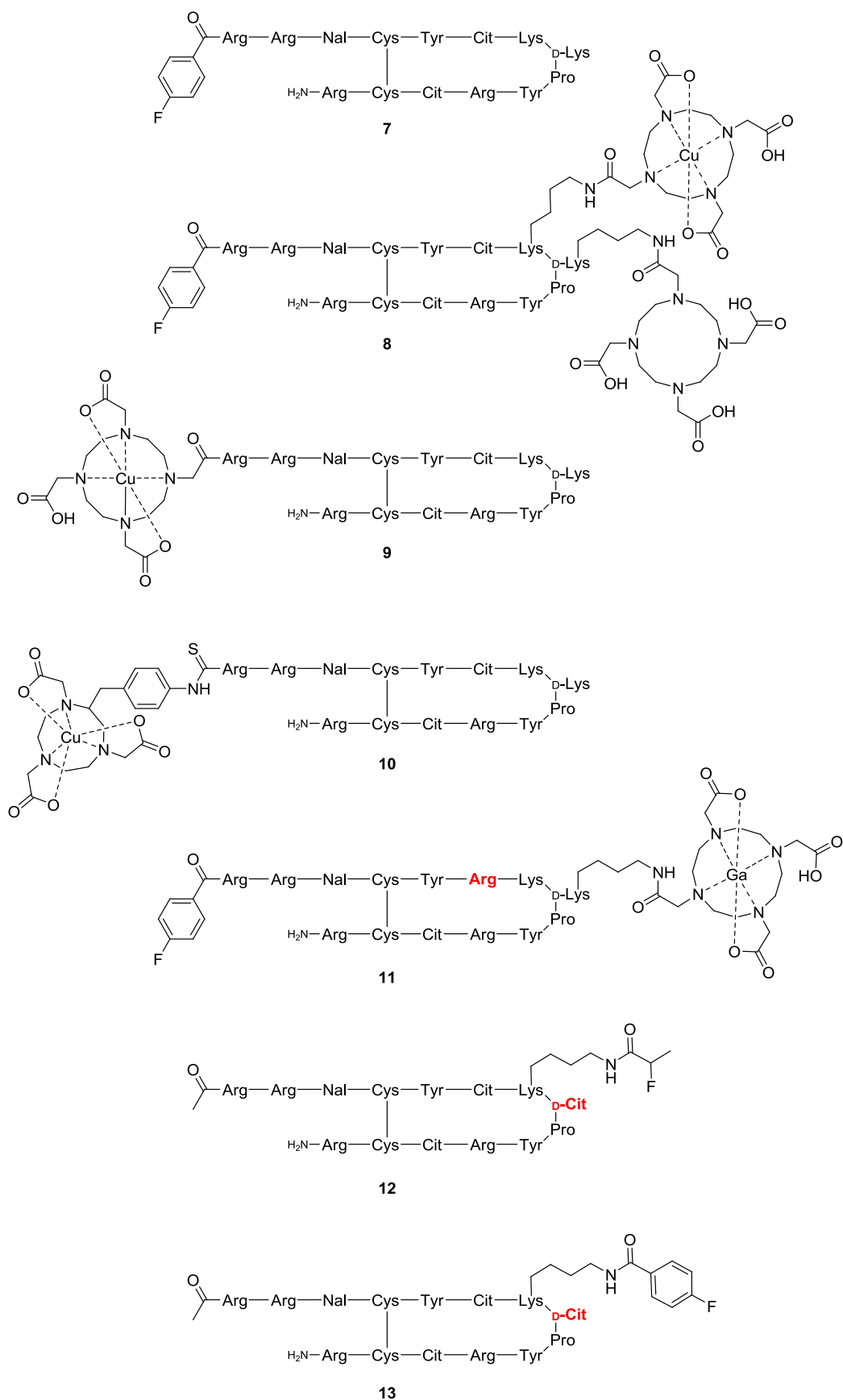


Figure 4 (continued). Compounds radiosynthesised as CXCR4-targeting PET imaging agents. In red are depicted modifications of the peptidic core from that of TN14003.

Table 1. Comparison of the radiochemical and imaging properties of the PET radiotracers designed to target CXCR4.

			Radiochemistry				Imaging				
Entry	Reference	Tracer	Δt^a (min)	RCY ^c (%)	Specific activity (GBq/ μ mol)	Starting activity (GBq)	Tumour model	Tumour uptake (% ID/g)	Positive -to- negative tumour ratio	Tumour -to- muscle ratio	Post- injection time- point ^d (h)
1	[95][22][96]	[⁶⁴ Cu] 1	60 ^b	–	134	0.74	CHO/CHO-XR4	12	8.8	59	6.0
							U87/U87-stb-CXCR4	30	6.1	47	1.5
2	[97]	[⁶⁴ Cu] 2	60 ^b	–	6.0	0.74	U87/U87-stb-CXCR4	96	22	363	1.5
3	[98]	[¹⁸ F] 3	–	–	–	–	–	–	–	–	–
4	[99]	[⁶⁸ Ga] 4	–	–	–	–	–/OH1	2.1	–	6.0	1.0
5	[100][101]	[⁶⁸ Ga] 5	45	25	22 ^b	1.3	–/OH1	6.2	–	17	1.0
6	[102]	[¹⁸ F] 6	150	10	21	1.1	healthy female BALB/c	–	–	–	–
7	[103]	[¹⁸ F] 7	150 ^b	5.8 ^b	7.0	–	CHO/CHO-XR4	2.3	1.4	2.9	3.0
8	[104]	[⁶⁴ Cu] 8	50	86	33	0.44	CHO/CHO-XR4	3.2	1.6	3.8	4.0
9	[105]	[⁶⁴ Cu] 9	50	91	37	0.44	CHO/CHO-XR4	5.0	5.0 ^b	39	4.0
10	[105]	[⁶⁴ Cu] 10	50	91	37	0.44	CHO/CHO-XR4	4.6	10 ^b	19	4.0
11	[106]	[⁶⁸ Ga] 11	35	51 ^b	29	0.50	–	–	–	–	–
12	[107]	[¹⁸ F] 12	150	3.1	18	5.9	CHO/CHO-XR4	4.3	4.6	high ^b	2.0
13	[107]	[¹⁸ F] 13	160	3.3	32	5.9	CHO/CHO-XR4	1.9	4.2	high ^b	2.0

^a Duration of the radiosynthesis, from the moment the radionuclide is produced. ^b Calculated or measured from the data available online. ^c Non-decay-corrected RCY. ^d Delay between the injection of the radiotracer and imaging.

From a radiolabelling perspective, it is clear that chelation of radiometals is fast and high yielding, with a radiosynthesis systematically shorter than 1 h and yields greater than 25% (entries 1, 2, 5, 8–11). Conversely, owing to the extended time necessitated to synthesise the prosthetic groups, radiofluorination strategies required ~2.5 h to afford the tracer in low RCYs (< 10%) (entries 6, 7, 12, 13).

In 2009–2012, small molecules AMD3100 and AMD3465 were radiolabelled with copper-64 into the resulting radiotracers [⁶⁴Cu]**1** and [⁶⁴Cu]**2** (entries 1, 2).^{22,95–97} They were assessed in Chinese hamster ovary (CHO) and glioblastoma (U87) cells, stably transfected with CXCR4 (CHO-XR4 at 6.8×10^5 receptors per cell and U87-stb-CXCR4 at 1.3×10^5 receptors per cell, respectively)¹⁰⁸ to afford the positive controls. Although high uptake in the tumours (up to 96% ID/g) and excellent contrast (tumour-to-muscle ratio of up to 363) were attained with the AMD3465 derivative, liver and kidney uptake were excessive (36 and 38% ID/g, respectively). Considering the relatively long half-life of copper-64 (12.7 h), this equates to high doses received by the patient's metabolic organs.

In 2011, a DOTA-containing dimer of cyclopentapeptide FC131 was labelled with gallium-68.⁹⁹ Tracer [⁶⁸Ga]**4** was used against a small cell lung cancer model (OH1), which endogenously expresses CXCR4 (entry 4). Good contrast was found, but no comparison with a negative tumour was performed. Moreover, uptake in spleen, lung and kidney, was superior to that observed for the tumour. Using the monomeric cyclopentapeptide improved tumour uptake by 3-fold (6.2% ID/g), although again these results were not compared to a negative cell line.^{100,101} The resulting tracer, [⁶⁸Ga]**5**, was stable *in vivo* over 30 min and showed low liver and spleen uptake (1.9 and 0.69% ID/g, respectively) (entry 5). This tracer is presently being evaluated in a phase I clinical trial.¹⁰⁹ Labelling of a precursor of similar structure with 4-[¹⁸F]fluorobenzaldehyde afforded tracer [¹⁸F]**6**, which showed very high rates of hepatobiliary and urinary excretion (entry 6).¹⁰² Although the tracer was not assessed with CXCR4-expressing tumour models, the non-existent uptake in endogenously CXCR4-expressing organs (*e.g.*, spleen, lung, bone marrow) was inauspicious.

Tetradecapeptide TN14003 has generated the most interest from the radiochemistry community. The first occurrence took the form of its *N*-4-[¹⁸F]fluorobenzoyl derivative in 2010.¹⁰³ The synthesis is reported as long and low yielding, and tracer [¹⁸F]**7** bound to red blood cells, therefore limiting blood circulation of free tracer (entry 7). This resulted in non-significant preference for CHO-XR4 over CHO cell lines, and higher uptake in numerous

organs (spleen, bone marrow, liver, kidney) than in CXCR4-expressing tumours. Derivatisation of both Lys7 and D-Lys8 with DO3A (which is DOTA attached to the peptide by one of its carboxylic acids) and labelling the resulting precursor with copper-64 has been unsuccessful as tracer [^{64}Cu]**8** presented similar behaviour towards red blood cells (entry 8).¹⁰⁴ On the other hand, attaching the chelating moiety to the peptide's *N*-terminus allowed preclusion of the red blood cell binding (entry 9 and 10).¹⁰⁵ The high uptake in liver and kidneys however resulted in high dose to metabolic organs. Recently, Zhang and co-workers labelled Ac-[D-Cit8]TN14003 with 2- ^{18}F fluoropropionate and 4- ^{18}F fluorobenzoate.¹⁰⁷ Tracers [^{18}F]**12** and [^{18}F]**13** showed satisfactory selectivity for CXCR4-expressing tumours over non-CXCR4-expressing and background tissues, although the very low yields (~3%) necessitated large amounts of starting activity (5.9 GBq) (entry 12, 13).

Finally, two radiolabelled molecules, one based on MSX-122 ([^{18}F]**3**, entry 3) and one based on TN14003 ([^{68}Ga]**11**, entry 11), were synthesised, although no *in vivo* studies were performed.^{98,106} In particular, compound [^{18}F]**3** was synthesised in order to confirm that SDF-1 α can block the binding of MSX-122 to CXCR4.

7. In this thesis

We were interested in the design and synthesis of PET radiotracers for the imaging of CXCR4-expressing tumours, and chose to work on three different classes of CXCR4 antagonists. The research conducted is presented in three chapters: chapter II describes efforts to design, synthesise and evaluate a tracer based on tetradecapeptide TN14003, while chapter III focuses on work on cyclic pentapeptides. Finally, synthetic efforts towards the development of a small molecule PET tracer are described in chapter IV.

CHAPTER II.

TETRADECAPEPTIDES

1. Design

1.1. From horseshoe crabs to tetradecapeptides

Tachyplesin I and polyphemusin II are antimicrobial peptides that participate in the defence mechanisms against invading microorganisms of the Japanese (*tachypleus tridentatus*) and the American (*limulus polyphemus*) horseshoe crabs, respectively (Figure 5).^{28,29}

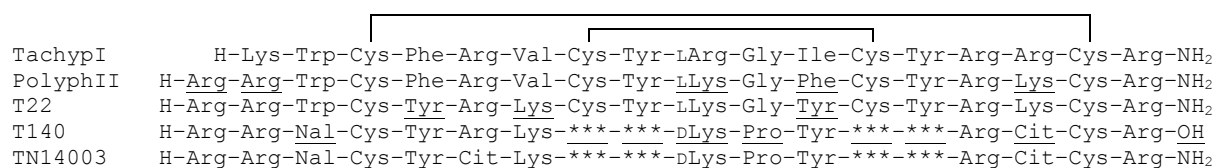


Figure 5. Comparison of the peptidic structures of tachyplesin I ("TachypI"), polyphemusin II ("PolyphII"), T22, T140 and TN14003. The disulfide bridges are depicted on top of the figure. Nal: 2-naphthylalanine. Cit: citrulline. ***: deleted amino acid. Underlined residues underline the differences with the structure just above.

Very rapidly after their discovery in 1988–1989, interesting anti-HIV activity was found for tachyplesin I, and its action seemed centred on the inhibition of virus adsorption.¹¹⁰ However, the selectivity of this compound was poor, presenting a half maximal cytotoxic concentration (CC₅₀) only 4 times higher than its half maximal EC₅₀. Attempts at structural modification of polyphemusin II afforded 40 analogues. One of these analogues, T22, differs from its parent peptide by residues on the 5-, the 7- and the 12-positions and has a selectivity index, defined as the ratio between the EC₅₀ value and the CC₅₀ value, of 170.^{111,112}

In 1997, Murakami and co-workers were able to prove that T22 targets CXCR4, which had been recently established as a co-receptor for entry of T-tropic strains of HIV-1.¹¹³ Further SAR analysis permitted the synthesis of T140,¹¹⁴ a smaller peptide made of 14 amino acids

with only one disulfide bridge present and presenting improved binding affinity. Nevertheless, T140 proved unstable in serum, undergoing cleavage of its C-terminal arginine residue, and its cytotoxicity was still challenging.¹¹⁵ Amidation of the C-terminus afforded a much more serum-stable peptide. Moreover, reduction of the total charge of the peptide by substituting Arg6 for a citrulline (Cit) drastically reduced the cytotoxicity, affording compound TN14003.

1.2. Strategies for peptide labelling peptides

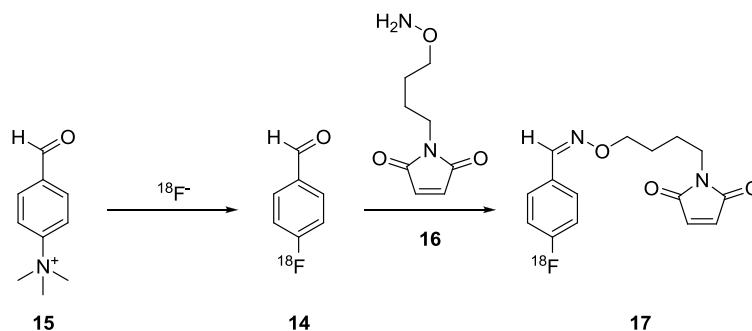
Because of their favourable pharmacokinetic properties (high affinity, fast tumour targetting, rapid excretion), peptides have been attractive for both radioimaging and radiotherapy. Amongst others, somatostatin, bombesin and RGD peptides were derivatised to offer PET and single-photon emission computed tomography (SPECT) tracers for various types of cancers such as prostate, breast or neuroendocrine tumours.⁹¹

The relative small size of these peptides results in rapid blood clearance with easy tumour penetration and therefore fast tumour targeting. Moreover, their synthesis is effortlessly amenable to solid-phase peptide synthesis, meaning that large arrays of analogues can be made by parallel and/or automated synthesis, thus allowing the engineering of tracers of improved binding affinity or metabolic stability by mere modification of certain amino acids. However, the choice of the radionuclide and the labelling position can be crucial for the tracer's characteristics,¹¹⁶ and if attained, nanomolar or sub-nanomolar potencies entail a requirement for high specific activities (see section III.2.3).¹¹⁷

Although fluorine-18 presents nearly ideal characteristics for PET, the use of this isotope for direct fluorination by displacement of triflate, tosylate or mesylate in the case of peptidic precursors is not well documented. Indeed, such electrophilic leaving groups are able to react with the other functional groups present in the peptide's side chains, which are more nucleophilic than fluoride. This would require the use of protecting groups, which in turn means additional purification and deprotection steps. Moreover, in polar aprotic solvent, [¹⁸F]fluoride is basic and can favour elimination over substitution, thus reducing the labelling efficiency.⁴²

As developed previously (see section I.5.2.3), these issues can be partially circumvented by the use of prosthetic groups. For instance, *para*-[¹⁸F]fluorobenzaldehyde **14**, synthesised by aromatic nucleophilic substitution of the corresponding trimethylammonium species **15**, can

be reacted with hydroxylamine **16** to afford cysteine-reactive prosthetic group **17** (Scheme 9).¹¹⁶



Scheme 9. Example of the synthesis of a cysteine-reactive prosthetic group.

However, this strategy requires numerous radiochemical steps and subsequent purifications, therefore reducing the overall RCY and final specific activity.¹¹⁸ Moreover, the specific activity can be further reduced if the bioconjugation of the prosthetic group does not allow significant difference of retention on HPLC columns.¹¹⁷ These features will be discussed in greater detail in section III.2.3.

On the other hand, the use of radiometal chelation is much more common in the field of peptide labelling. A variety of chelating agents have been devised over the past decades, allowing for fast irreversible labelling with positron emitters for PET (*e.g.*, ^{64}Cu , ^{68}Ga) or γ -emitters for SPECT (*e.g.*, $^{99\text{m}}\text{Tc}$, ^{111}In).⁹⁰ Although such chelating groups are relatively large, their conjugation to a peptide can have limited consequences on the binding affinity provided that the position of the derivatisation is carefully chosen.⁹¹ On top of this, the labelling reaction is single-step, generally, fast and does not normally require subsequent HPLC purification but only solid-phase extraction. Chelation can generally also be achieved with lower quantities of starting material compared to standard organic chemistry radiolabelling strategies, thus affording the tracer in relatively high specific activity.

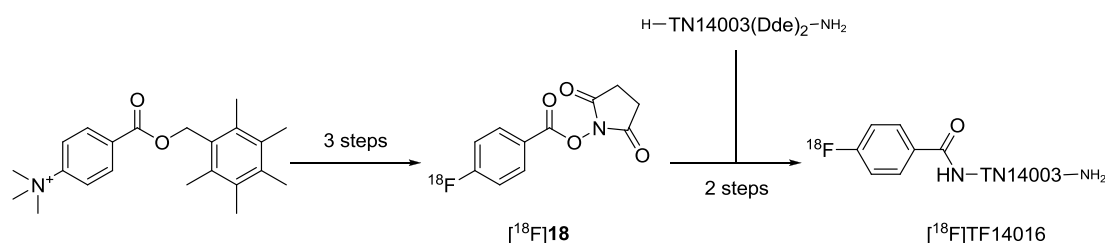
It is worth noting that an intermediate solution for peptide labelling consists of forming inorganic fluorine–metal bond for the incorporation of a radionuclide in a precursor, thus exploiting the excellent PET isotope properties of fluorine-18 (97% β^+ decay, $t_{1/2} = 110$ min, low energy) and from the ease of labelling by chelation (aqueous conditions, no HPLC

purification). For instance, formation of Si–F and B–F bonds have been used in order to radiolabel peptides with fluorine-18,^{119,120} but these strategies gave rise to potential issues of defluorination, as evidenced by high bone uptake. Conversely, McBride and co-workers^{118,121} recently developed a method to form aluminium [¹⁸F]fluoride complexes of NOTA-containing peptide, with no apparent sign of defluorination, thus affording a practical way to introduce fluorine-18 into a peptide.

1.3. Existing tetradecapeptidic radiolabelled probes for CXCR4

Although potent, peptide TN14003 was found to be unstable in rat liver homogenate by cleavage of Arg1–Arg2–Nal3 sequence from the *N*-terminus.¹²² Modification of the *N*-terminus by addition of an *N*^α-4-fluorobenzoyl group afforded analogue TF14016, which displayed excellent mouse serum stability. Its resistance against rat liver homogenate degradation (~75% recovery after 2 h) was found adequate for PET as it allows stability of the molecule over the time of the scan and relatively rapid degradation afterwards. The capping of the *N*-terminus also allowed an improvement of the binding affinity for CXCR4.¹²³

Jacobson and co-workers undertook the radiosynthesis of [¹⁸F]TF14016 (Scheme 10).¹⁰³



Scheme 10. Jacobson's synthesis of [¹⁸F]TF14016.¹⁰³

Activated ester [¹⁸F]18 was synthesised in three steps and used to label a derivative of TN14003 in which the side chain's amine groups of both Lys⁷ and D-Lys⁸ were protected with [1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] (Dde). Once labelled, the peptide had to undergo removal of Dde protecting groups. Disappointingly, this long (~3 h from aqueous [¹⁸F]fluoride) and low yielding (< 10% DC-RCY) synthesis afforded a tracer that bound non-specifically to red blood cells, thus increasing the background uptake. Although it was found that co-injection of TF14016 improved the tumour-to-muscle ratio from 2.9 to 22 and allowed

visualisation of the tumour, this was not deemed adequate for the clinic, as injection of pharmaceutical quantities of CXCR4 antagonists potentially has some adverse effects.

Attempts at derivatising TF14016's scaffold to allow labelling with copper-64 were carried out by the same group in 2011.¹⁰⁴ In this case, the side chain's amine groups of both Lys⁷ and D-Lys⁸ were coupled to DOTA for chelation with the radiometal. The choice of these positions for modification is in accordance with what was described by Wu *et al.*¹², namely that CXCR4 co-crystallised with CVX15, an analogue of TN14003 [CVX15 = TN14003–Gly–D-Pro–OH], in a binding mode in which both the *N*- and the *C*-termini are buried in the helix bundle while the peptide's turn protrudes out of the receptor, towards the extracellular milieu. The primary purpose of Jacobson's design was met as the radiolabelling could be performed efficiently and in short amounts of time without the need of HPLC purification. However, the tracer once more showed non-specific binding to red blood cells. As earlier, co-injection with the precursor increased the tumour-to-muscle ratio, although to a lesser extent than for [¹⁸F]TF14016. A similar approach involving bioconjugation of only one DOTA chelating group at TN14003's D-Lys⁸ was described elsewhere,¹⁰⁶ although no *in vivo* data was reported.

More recently, Jacobson *et al.*¹⁰⁵ published the radiolabelling with copper-64 of another analogue of TN14003 in which the *N*-terminus was coupled to DOTA (by one of the chelating agent's carboxylic acid) or to a NOTA derivative (by reaction with an isothiocyanate functional group) (Figure 6).

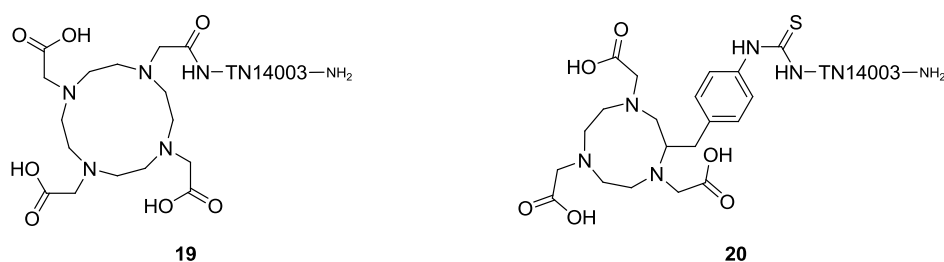


Figure 6. Structures of the tracers developed by Jacobson *et al.* based on derivatisation of peptide TN14003 at its *N*-terminus to allow chelation of copper-64.

The affinity for CXCR4 exhibited by DO3A- and NOTA-Bn-thiourea- bioconjugates were comparatively weaker than the original TN14003. It is rather surprising than any affinity

could be measured as both the *C*- and *N*-termini of CVX15 are buried within CXCR4's helix bundle.¹² This result shows that either such peptides are able to bind in two different poses (with either the *C*- and *N*-termini or the turn buried in the binding pocket) or that there is enough room within the binding pocket to accommodate such large chelating groups. Keeping in mind that TN14003 is shorter by two amino acids than CVX15, this second hypothesis seems plausible. This notion is further supported by the difference of binding affinity between **19** and **20**, suggesting steric hindrance towards TN14003's *N*-terminus. Interestingly, the tracers prepared from these two precursors showed no non-specific binding to red blood cells. However, even with the *N*-terminus protected from metabolic cleavage by the DO3A or NOTA derivative, high metabolism activity was found.

1.4. Modifications to TN14003

Tracers [⁶⁴Cu]Cu–**19** ([⁶⁴Cu] **9**) and [⁶⁴Cu]Cu–**20** ([⁶⁴Cu]**10**) showed interesting biological characteristics as modification of TN14003 at the *N*-terminus with a chelating group seemed to eliminate the non-specific binding to red blood cells encountered with other tracers based on this peptide.¹⁰⁵ Nevertheless, the combination of high metabolism rate and ⁶⁴Cu's long half-life leads to a long time of residence for the radioactive tracer in metabolic organs (namely, liver and kidneys), thus delivering a high dose to the patient.

The design of a new PET probe that would circumvent these problems was therefore undertaken. By first performing the radiolabelling of the probe with ⁶⁸Ga instead of ⁶⁴Cu, the time of residence in metabolic organs was anticipated to be considerably decreased, the half-life of ⁶⁸Ga being more than ten times lower than that of ⁶⁴Cu. However, a consequence of this is also the short amount of time allowed between the injection of the tracer and the scan. It is usual to start scanning a few hours after injecting a copper-64-labelled tracer: this allows time for the body to wash out non-specific binding, thus affording lower background and improved contrast. Although the patient would undeniably benefit from shorter clinical procedure concomitant with gallium-68, this is a further challenge for the tracer.

Removal of the benzyl thiourea linker between the peptide and the NOTA chelating group could play in our favour as the importance of the modifications to TN14003 is likely to affect the binding affinity of the tracer. Thus, a smaller modification of TN14003 could result in improved affinity of the tracer for the receptor and therefore superior tumour-to-background ratio. Furthermore, by eliminating potential sites for metabolism, a reduction of the observed liver uptake is anticipated, allowing for increased uptake in the tumour.

Taking into consideration the above, compound **21** was devised for labelling with gallium-68 (Figure 7).

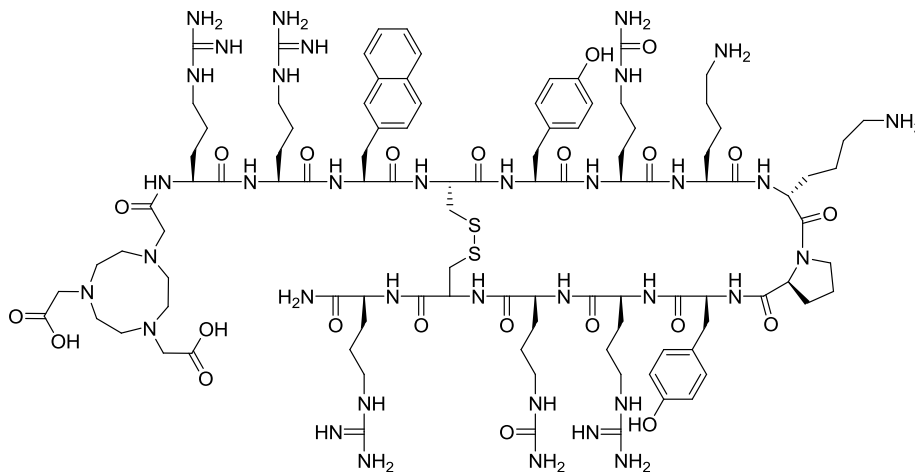


Figure 7. Structure of **21**.

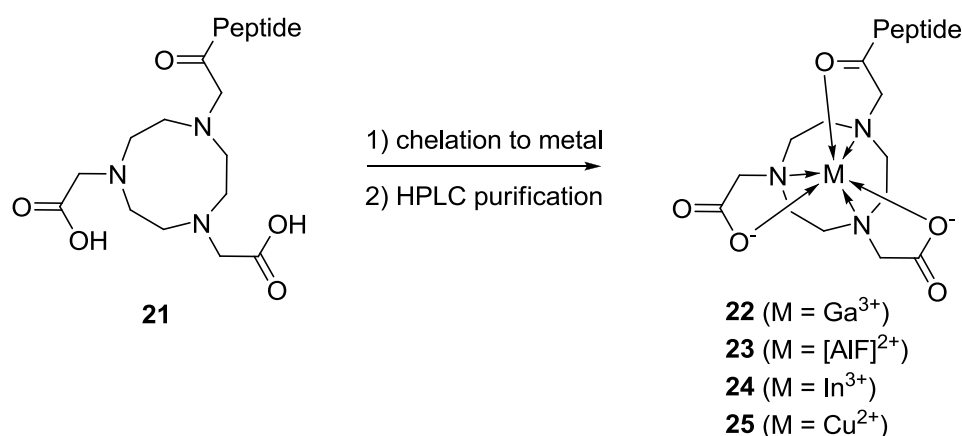
In this structure, the TN14003 peptidic core was conserved in order to benefit from its potent affinity to CXCR4. A NO2A group, coupled to the peptide's *N*-terminus, would allow chelation of gallium-68 with minimal modifications to the core structure, hopefully allowing low binding potency loss, limited metabolism sites and therefore shorter exposure of the subject to ionising radiation.

2. Reference compounds

2.1. Synthesis of various chelates

Compound **21** was obtained from a commercial source. Its chelation with Ga^{3+} , In^{3+} , Cu^{2+} and $[\text{AlF}]^{2+}$ was considered to allow for exploration of the influence of the nature of the metal on binding affinity. Simultaneously, this approach would allow screening of various radiolabelling isotopes for SPECT imaging (with $^{111}\text{In}^{3+}$) and PET imaging (with $^{68}\text{Ga}^{3+}$, $^{18}\text{F}[\text{AlF}]^{2+}$ and $^{64}\text{Cu}^{2+}$).

The gallium chelate was obtained upon mixture of precursor **21** with a GaCl_3 solution in sodium acetate buffer (Scheme 11 and Table 2).¹²⁴

Scheme 11. Chelation of various non-radioactive metals to precursor **21**.Table 2. Reaction conditions and yields for the chelation of various non-radioactive metals to precursor **21**. Reactions were run on a 3.09–3.32 mg scale.

Entry	Compound	Metal source (equiv.)	Solvent	Reaction temperature	Reaction duration	Yield
1	22	GaCl ₃ (3.0 equiv.)	NaAB ^a	80 °C	10 min	62%
2	23	AlCl ₃ (3.0 equiv.) + NaF (3.3 equiv.)	NaAB ^a	100 °C	15 + 15 min ^c	54%
3	24	InCl ₃ (10.0 equiv.)	AAB ^b	80 °C	60 min	28%
4	25	CuCl ₂ (10.0 equiv.)	AAB ^b	r.t.	120 min	>99%

^a sodium acetate buffer (pH 4.0, 0.1 M); ^b ammonium acetate buffer (pH 6.0, 65 mM); ^c AlCl₃ and NaF were first reacted at 100 °C for 15 min, then precursor **21** was added and the mixture was stirred at 100 °C for 15 min.

Similarly, the indium-, copper- and aluminium fluoride chelates were obtained following literature procedures.¹²⁴⁻¹²⁶ The various chelates were purified by semi-preparative HPLC affording the compounds in their pure forms as trifluoroacetate (TFA) salts in modest to quantitative yields. The difference in yields can be explained by the ease of manipulating the products after lyophilisation: for instance, analogue **25** was a compact non-electrostatic easily transferable solid, while analogue **24** was a light electrostatic solid. All analogues were characterised by mass spectrometry. Although the molecular peak could never be detected,

the +3, +4 and +5 ions (as well as the +2 and +6 ions for some analogues) were observed. Furthermore, ^{71}Ga nuclear magnetic resonance (NMR) spectroscopy presented two peaks for **22**, corresponding to the two possible chelate isomers (Figure 8), while ^{19}F NMR spectroscopy of **23** presented a peak at $\delta = -122.5$ ppm, bringing additional confirmation of the identity of these two chelates.

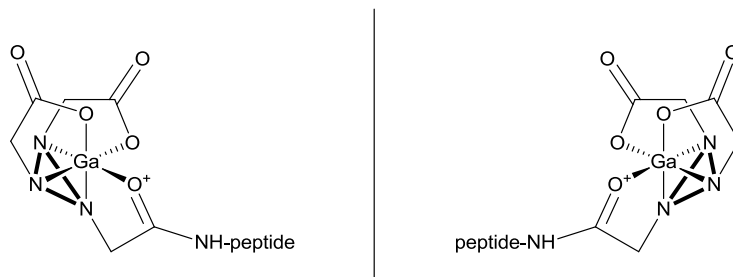


Figure 8. Illustration of the two isomers of a Ga–NOTA chelate.

2.2. Preliminary *in vitro* biological evaluation

We first set out to confirm that our NO2A conjugate, **21**, and its chelates with various metal cations, have similar biological properties to those described previously in the literature. To this end, we decided to compare the antagonism of these molecules against CXCR4 to that of TC14012, a commercially available TN14003 analogue of comparable binding potency (literature: TC14012 = [D-Cyt⁸]TN14003, $\text{IC}_{50} = 19.3$ nM; TN14003, $\text{IC}_{50} = 12.4$ nM).¹¹⁵ The affinities of our antagonists for CXCR4 were assessed for their ability to inhibit [^{125}I]SDF-1 α 's binding and to inhibit chemotaxis (Figure 9 and Table 3).

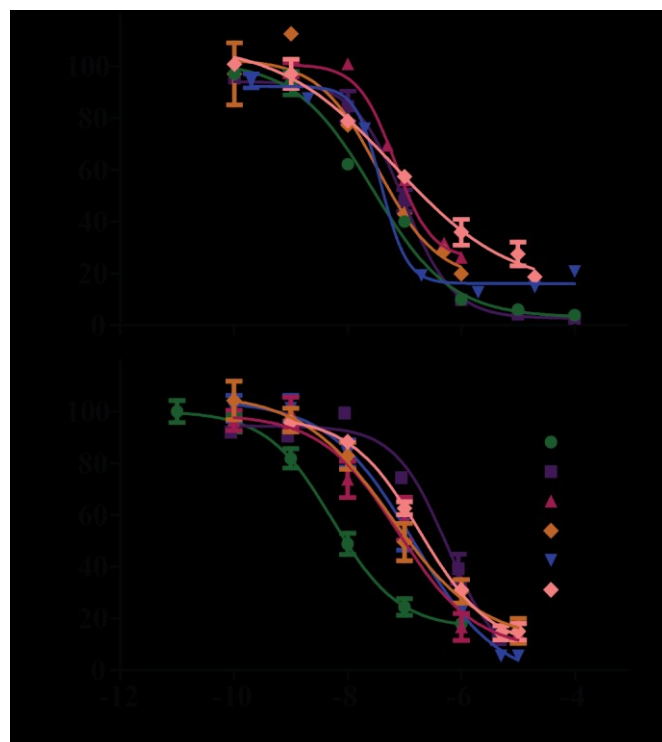


Figure 9. Comparison of the affinities of TC14012, **21** and our four chelates with two methods. A: Radioligand competitive binding assay. B: Migration assay.

Table 3. Comparison of the affinities of TC14012, **21** and our four chelates by radioligand competitive binding assay and migration assay.

Entry	Compound	Structure	IC ₅₀ (nM)	
			Binding	Migration
1	TC14012	[D-Cyt ⁸]TN14003	23.1±11.1	4.9±0.8
2	21	NO2A–TN14003	65.2±20.2	423±95.5
3	22	Ga ³⁺ –NO2A–TN14003	65.8±32.9	60.8±18.6
4	23	[AlF] ²⁺ –NO2A–TN14003	66.3±30.8	53.6±19.1
5	24	In ³⁺ –NO2A–TN14003	26.1±11.1	107±41.1
6	25	Cu ²⁺ –NO2A–TN14003	63.0±28.3	220±103

Data are **mean** ± SD of three independent experiments

The competitive binding assay showed that the bioconjugation of the NO2A chelating moiety to TN14003's *N*-terminus had very limited influence on the binding potency. Moreover, the

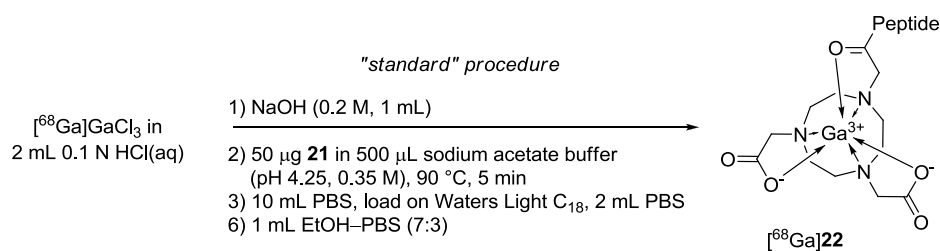
chelation of the precursor to gallium(III), aluminium(III) fluoride, indium(III) or copper(II) had no or marginal measurable influence. On the other hand, the downstream effects were substantially stronger as revealed by the migration assay results. For instance, precursor **21** was 100-fold less chemotactic than TN14012. Also, while the gallium- and aluminium fluoride- chelates improved the chemotacticity of the precursor approximately 7-fold, chelation of the larger metal cations indium(III) and copper(II) has less effect (2–4-fold lower IC_{50}). The narrow distribution of mean values along with the relatively sizeable standard deviations render difficult any attempt at drawing definite conclusions about the biological explanation for the differences between the two assays. In addition, the migration assay measures a downstream effect of the inhibition of CXCR4, which is only partially related to our primary objective, namely designing a molecule that binds to CXCR4. Therefore, the binding assay was considered to afford results of greater significance and reliability for our purpose.

As our gallium-chelate, **22**, showed potent binding activity, we decided to carry on with the radiosynthesis of this tracer.

3. Optimisation of the radiolabelling procedure

3.1. Initial attempts

Typically, the experiments were performed as follows: [^{68}Ga]GaCl₃ was obtained from the gallium generator in ~2 mL of a 0.1 N HCl aqueous solution into a sealed 5-mL Wheaton vial *via* a steel needle. This eluate was then quenched with sodium hydroxide under stirring, and a solution of 50 µg of precursor **21** in 500 µL of sodium acetate buffer (pH 4.25, 0.35 M) was added. The reaction was allowed to stir for a given time and an aliquot of the reaction mixture was taken to monitor the conversion rate by radio-HPLC, and reported as the ratio between the area under the peak corresponding to the tracer and the total area under the curve. The reaction mixture was then diluted with 10 mL of PBS and the resulting solution was passed through a Waters Light C₁₈ SPE cartridge. The cartridge was washed with 2 mL of PBS, dried with 20 mL of air and eluted with 1 mL of EtOH–PBS (7:3). The radioactivity left in the reaction vessel ("Wheaton"), in the solution coming from the loading of the diluted reaction mixture onto the SPE cartridge and subsequent washing ("Wash"), in the cartridge after elution ("Cartridge") and in the SPE eluate ("SPE eluate") were measured, decay-corrected, and reported in Table 4 as their fraction to the starting amount of radioactivity.

Table 4. Scheme and results of various attempts at performing labelling of precursor **21**.

Entry	Reaction conditions			Reaction monitoring		Radioactivity reading			
	Use of NaOH (Y/N)	Precursor concentration /g.L ⁻¹	Reaction temperature / $^\circ\text{C}$	Reaction duration /min	Conversion $\times 100$	Wheaton $\times 100$	Wash $\times 100$	Cartridge $\times 100$	SPE eluate $\times 100$
1 ^a	N	0.1	110	15	15	1.5	83	1.4	12
2	Y ^b	0.1	110	15	62	12	43	17	27
3	Y ^b	0.1	110	15	59	2.0	38	2.5	57
4	Y ^b	0.1	110	25	6	—	—	—	—
5	Y ^b	0.1	110	10	65				
6				20	53				
7				30	46	38	29	24	8.6
8	Y ^b	0.1	90	5	62				
9				15	60				
10				25	58	57	34	5.3	3.1

For clarity, entries corresponding to one single gallium elution were alternately shaded. ^a The precursor was dissolved in 100 μL of 3.5 M acetate buffer; ^b injected as 100 μL of 2 N $\text{NaOH}(\text{aq})$ in one portion.

A first attempt at labelling the precursor using 100 μL of 3.5 M sodium acetate buffer (pH 4.25) to quench the 2 mL of 0.1 M $\text{HCl}(\text{aq})$ coming from the generator without the use of $\text{NaOH}(\text{aq})$ afforded only 15% conversion after 15 min at 110 $^\circ\text{C}$ (entry 1). Repetition of this attempt showed no improvement of the conversion rate (data not shown). Although the

reaction mixture reached the pH considered optimal for gallium labelling, this low conversion was thought to result from either acetate chelating to gallium(III) and therefore slowing down its incorporation in precursor **21**, or from a too high quantity of contaminants coming from the acetic acid or the sodium acetate used to make the buffer. As a result, the strength of the sodium acetate buffer was reduced 10-fold. Nevertheless, because this new buffer would not be able to quench 0.2 mmol of HCl, NaOH_(aq) needed to be added to the generator eluate before adding the solution of precursor in sodium acetate buffer.

These modifications improved the conversion to ~60% in 15 min, supporting our initial hypotheses (entry 2–3). Leaving the reaction mixture to stir longer surprisingly reduced the conversion drastically to 6% (entry 4), suggesting that the desired complex was unstable at 110 °C. This was confirmed, although to a lesser extent, by monitoring the conversion at different time-points (entry 5–7). Reducing the reaction temperature to 90 °C showed improved stability of the complex with negligible expulsion of chelated gallium-68 (entry 8–10). However, the conversion was still sub-optimal. It was noticed in particular that such average rates seemed to correlate to the unexpectedly high amounts of radioactivity found in the Wheaton vial after emptying it and washing it with 2×5 mL of PBS and in the Waters Light C₁₈ cartridge used for purification. Indeed, it was not anticipated that both [⁶⁸Ga]GaCl₃ and [⁶⁸Ga]**22** would be insoluble in water.

3.2. Considerations about gallium colloid and metastability

It has been reported that the formation of gallium colloid is a process witnessed when radiolabelling with gallium-68¹²⁷ and that this can be circumvented by the use of sodium acetate buffer.¹²⁸ Between approximately pH 3 and 7, gallium(III) forms a hydroxide precipitate, Ga(OH)₃, which is scarcely soluble in water. The formation of such colloid accounts for the results seen in Table 4: precipitation of [⁶⁸Ga]Ga(OH)₃ reduces the amount of soluble forms of gallium complexes, thus reducing the overall formation of [⁶⁸Ga]**22**. This is not directly noticeable by monitoring the reaction mixture, as [⁶⁸Ga]Ga(OH)₃ is not present in the injected sample, which explains why the "Conversion" values scarcely correlate to the "SPE eluate" values. This also accounts for the radioactivity left in the Wheaton vial after thorough washing ([⁶⁸Ga]Ga(OH)₃ adsorbs to the vial's walls) and the radioactivity left in the SPE cartridge (the colloid is filtered by the SPE cartridge and is not eluted). Two test reactions (data not shown) were performed by using acidified or basified PBS to dilute the reaction mixture; only negligible amounts of radioactivity on the Wheaton vial's wall (2–

6.6%) or on the cartridge after elution (2.5–6.2%) were detected, which can be explained by the resolubilisation of $[^{68}\text{Ga}]\text{Ga}(\text{OH})_3$ under acidic or basic conditions.

In order to confirm this hypothesis, the solubility of $\text{Ga}(\text{OH})_3$ in our conditions was calculated and compared it to the amount of gallium present in no-carrier-added radiolabelling experiments. Thus, the equation relating the activity A of a sample to the number of atoms of gallium-68 N_{Ga} and their half-life $t_{1/2}$ is:

$$A = N_{\text{Ga}} \times \frac{\ln 2}{t_{1/2}} \Leftrightarrow n_{\text{Ga}} = \frac{N_{\text{Ga}}}{N_A} = \frac{A \times t_{1/2}}{N_A \times \ln 2}$$

where N_A is Avogadro's number. Applied to the generator's eluate (typically, 1 mCi/mL), the total concentration of gallium atoms is $[\text{Ga}^{\text{III}}] = 3.6 \times 10^{-10} \text{ M}$.

Then, with a soluble product constant of $K_{\text{sp}} = 7.3 \times 10^{-36}$, the solubilisation of $\text{Ga}(\text{OH})_3$ gives the following formulae:



$$K_{\text{sp}} = 7.3 \times 10^{-36} \approx x \times (10^{\text{pH}-14})^3$$

for an efficiently buffered system, where x is the advancement of the reaction of resolubilisation of $\text{Ga}(\text{OH})_3$. As a result, the maximal concentration of gallium in solution at pH 4.25 is:

$$x = 1.3 \times 10^{-6} \text{ M} > 3.6 \times 10^{-10} \text{ M}$$

Therefore, theoretically, all gallium atoms should be solution for a 1 mCi.mL⁻¹ elution. However, the presence of contaminating metals in solution may co-precipitate with $\text{Ga}(\text{OH})_3$. Among such contaminants, zinc-68, the daughter isotope of gallium-68, is eluted from the generator along with gallium-68. The amount of zinc-68 can be estimated as follows. Consider a generator in a steady state of 4.0 mCi of ^{68}Ga , *i.e.* containing 1.4×10^{-12} mol of ^{68}Ga . In one half-life, half of the radioisotopes would have decayed to ^{68}Zn , thus producing 7.0×10^{-13} mol of ^{68}Zn every 67.7 min. This corresponds to an increase of $7.5 \times 10^{-9} \text{ M}$ of ^{68}Zn in 2 mL generator eluate every 24 h.

Other sources of metal contamination are gallium-68's parent isotope ^{68}Ge , the matrix on which ^{68}Ge and ^{68}Ga are adsorbed (TiO_2 or SnO_2), the generator's output needle (steel) and any trace contamination of the buffer, the precursor, the reaction vial or the stirbar.

Therefore, although the amounts of radioactive gallium involved are theoretically low enough to be soluble despite the very low solubility of $\text{Ga}(\text{OH})_3$, the conjunction of the above metal contaminants is expected to result in the precipitation of gallium during the labelling step. However, under certain conditions, it is possible that the kinetics of the formation of $\text{Ga}(\text{OH})_3$ could be slow enough to allow time for stabilisation of $^{68}\text{Ga}^{3+}$ into the chelating moiety such as NO2A. Furthermore, sodium acetate was reported to allow, with the weak chelation of acetate anions to gallium(III), to protect gallium from precipitation.¹²⁸ This should pull the reaction of resolubilisation of $\text{Ga}(\text{OH})_3$ towards the formation of solubilised gallium.

Following the hypothesis that soluble gallium should be metastable during the quenching step, we attempted to reduce the disturbance caused by this step by injecting a more dilute quantity of sodium hydroxide to the generator's eluate. Considering that the injection of 100 μL of 2 N $\text{NaOH}_{(\text{aq})}$ solution in one lump to the acidic solution of $[\text{}^{68}\text{Ga}]\text{GaCl}_3$ would result in the formation of locally strongly basic spot of solution, thus encouraging the formation of gallium colloid, we attempted to quench the acidity more gently with a more dilute solution of $\text{NaOH}_{(\text{aq})}$ (Table 5).

This resulted in a generally improved incorporation of gallium into the NO2A chelating group (entry 1–7) although reproducibility was low (entry 8–11), occasionally exhibiting lower conversion rate along with formation of gallium colloid. Attempts at changing the amount of precursor did not afford improvements in the reproducibility (entry 12–23).

Addition of sodium citrate to the reaction mixture has been reported to help keep gallium(III) in solution.¹²⁷ However, in our hands, two attempts at adding sodium citrate to the generator's eluate in order to stabilise ^{68}Ga were carried out. Used in a large excess, sodium citrate dramatically reduced the formation of gallium colloid, but prevented chelation of gallium to the precursor (entry 24). Reducing the amount of citrate resulted in the formation of a substantial amount of gallium colloid and no tracer product formed (entry 25).

Table 5. Result of further attempts at performing labelling of precursor **21**.

Entry	Reaction conditions			Reaction monitoring		Radioactivity reading			
	Use of NaOH (Y/N)	Precursor concentration /mg.mL ⁻¹	Reaction temperature /°C	Reaction duration /min	conversion ×100	Wheaton ×100	Wash ×100	Cartridge ×100	SPE eluate ×100
1	Y ^a	0.1	90	5	97				
2				15	92	18	46	3.1	28
3	Y ^a	0.1	90	5	97				
4				15	91	4.0	44	4.8	22
5	Y ^a	0.1	90	5	97				
6				15	91	6.6	71	6.2	15
7	Y ^a	0.1	90	5	99	0.6	0.9	2.9	44
8	Y ^a	0.1	90	5	75	21	6.7	26	32
9	Y ^a	0.1	90	5	47	38	3.9	56	3.0
10	Y ^a	0.1	90	5	8	29	5.6	66	2.1
11	Y ^a	0.1	90	5	0	38	10	53	0.0
12	Y ^a	1.0	90	5	93	26	5.2	4.7	26
13	Y ^a	0.4	90	5	96	18	4.1	11	55
14	Y ^a	0.2	90	5	97	16	2.3	26	50
15	Y ^a	0.2	90	5	97	25	2.3	11	56
16	Y ^a	0.04	90	5	96	29	2.5	12	48
17	Y ^a	0.04	90	5	97	8.5	2.6	16	59
18	Y ^a	0.04	90	5	0	—	—	—	—
19	Y ^a	0.04	90	5	93	17	13	39	34
20	Y ^a	0.04	90	5	0	—	—	—	—
21	Y ^a	0.04	90	5	84	18	13	30	24
22	Y ^a	0.04	90	5	50	36	24	25	12
23	Y ^a	0.04	90	5	0	—	—	—	—
24	Y ^b	0.04	90	5	0	0.3	93	5.7	0.0
25	Y ^c	0.04	90	5	0	38	36	25	0.0

For clarity, entries corresponding to one single gallium elution were alternately shaded. ^a Slowly injected as ~1 mL of 0.2 N NaOH(aq) to pH ~ 3–4 into a stirring Wheaton vial; ^b preceded by the addition of 100 µL of 0.1 M of phosphate-citrate buffer; ^c preceded by the addition of 100 µL of 10 µM of phosphate-citrate buffer.

3.3. Simplification of the labelling procedure

Our aforementioned attempts at reducing the perturbation undergone by gallium-68 were relatively successful as they enabled higher labelling rates but with low reproducibility. The low reproducibility was therefore thought to be related to the complexity of the procedure, entailing a large number of variables influencing the outcome of the labelling reaction. For instance, several variables coming from the use of a stirbar could be its shape, its cleanliness or the speed of stirring. Similarly, the quenching step with NaOH could be influenced by the molarity of the solution, the volume added and the rate of quenching. Furthermore, the low quantities and high dilutions dealt with might result in a plethora of other undefined variables.

Thus, our procedure was amended with the aim of drastically reducing the number of stages necessary to carry out the radiolabelling of precursor **21**, and therefore the number of possible variables influencing the outcome of this procedure. For this purpose, the procedure was simplified to what is strictly necessary: the elution of the generator, the heating of the reaction mixture, and its dilution with PBS in order to trap the product onto the SPE cartridge. In parallel, we endeavoured to limit metal contamination (Table 6).

Thus, by changing the needle used to dispense the generator's eluate into the Wheaton vial before each elution, we anticipated that the ageing of the needle could be eliminated as a variable, possibly reducing the amount of iron in the reaction mixture. Furthermore, a stronger control of the cleanliness of the reaction mixture by using an acid bath to wash the vial and by not using a stirbar were expected to reduce the propensity of gallium colloid to form. Finally, simplification of the buffering step by having it controlled by the gallium generator in one step rather than controlling it manually in two steps (addition of NaOH followed by addition of sodium acetate buffer) was anticipated to greatly improve the reproducibility of this step. To do so, we decided to use a stronger buffer in a larger volume and made sure that the final pH of the reaction mixture brings the reaction mixture to the buffer's optimal pH. Thus, the use of 2.0 mL of sodium acetate buffer at 0.5 M and pH 5.8 generates comfortable margin in the volume eluted by the gallium generator (1.5–6.7 mL) to attain pH 4.8 ± 0.5 .

In practice, this resulted in a more controlled formation of gallium colloid and an improved conversion rate (Table 7).

Table 6. Simplification of the labelling procedure.

Initial procedure	Simplified procedure	Examples of variables affected
Use of the same needle for the gallium-generator	Use of a new needle for the gallium-generator	Ageing of the needle Quantity of metal contaminants in reaction mixture
Wheaton vial washed with soapy water, Milli-Q water, acetone and oven-dried	Wheaton vial in HNO ₃ bath for 24 h, rinsed with MQ water and oven-dried	Quantity of metal contaminants in the reaction mixture
Use of a stirbar	No stirbar	Presence of contaminants from stirbar Speed of stirring
Elution in an empty Wheaton vial	Elution in sodium acetate buffer directly	Reproducibility of the buffering process (controlled manually in 2 steps vs. controlled by the Eckert-Ziegler automaton in 1 step)
Quenching with NaOH	No quenching with NaOH	
Addition of precursor and sodium acetate buffer	—	

Table 7. Differences between the simplified and the initial labelling procedures.

Procedure	Conversion	Wheaton	Wash	Cartridge	DC-RCY	Repeats
Simplified	90±4%	7±3%	7±3%	7±3%	58±6%	13
Initial	65±41%	21±13%	24±27%	24±21%	21±17%	16

Note: DC-RCYs do not correspond to the same measurement in both cases; for the initial procedure, it corresponds to the totality of the cartridge elution with 1 mL of EtOH–PBS (7:3); for the simplified procedure, it corresponds to the second 150-μL fraction of the cartridge elution with EtOH–PBS (7:3).
Data are **mean** ± SD.

Thus, the amount of gallium colloid formed during the radiosynthesis was reduced from 69% to 22% (Wheaton+Wash+Cartridge) while the conversion was increased to 90%.

Consequently, the amount of product collected was improved by approximately 3-fold. On top of this, the simplified procedure resulted in a much more reliable radiolabelling step as illustrated by the standard deviations in Table 7.

Finally, the new procedure afforded the tracer in 11 ± 1 min from elution to delivery in high radiochemical purity ($98 \pm 2\%$) and an apparent specific activity of $10.5 \text{ GBq} \cdot \mu\text{mol}^{-1}$ starting from 230 MBq of aqueous $[^{68}\text{Ga}]\text{GaCl}_3$ (Figure 10).



Figure 10. Quality control of the delivered $[^{68}\text{Ga}]\mathbf{22}$. A: radioactive trace of the formulated tracer. B: UV chromatogram of the formulated tracer, co-injected with the non-radioactive reference compound.

The tracer was stable over 4 h in 10% (v/v) EtOH–PBS and the logarithm of its distribution coefficient between *n*-octanol and PBS was measured to be -3.58 ± 0.15 ($n = 5$).

4. Preclinical tracer validation

4.1. Cellular uptake assay

Cellular uptake of [^{68}Ga]**22** was assessed in U87.CD4 cells transfected with CXCR4 (U87.CD4.CXCR4) (Figure 11).



Figure 11. [^{68}Ga]**22** *in vitro* uptake in U87.CD4 and U87.CD4.CXCR4 cells. Data are mean $\pm$ SEM of three independent experiments and statistical significance was confirmed using a 2-way ANOVA with Bonferroni post-tests ($P < 0.05$).

Gratifyingly, 60-min cellular uptake of [^{68}Ga]**22** showed a four-fold higher uptake in the CXCR4-expressing cells compared to isogenic CXCR4-low cells, confirming potency of the tracer to CXCR4 as seen in the competitive binding assay. Furthermore, pre-incubation of the cells with 20 μM of non-radioactive **22** showed a decrease of the cellular uptake to the level of the CXCR4-low cells control, demonstrating specificity of the tracer for CXCR4. These biological data, along with the IC_{50} value measured for our tracer, encouraged us to assess the tracer *in vivo*.

4.2. Positron emission tomography

U87.CD4 and U87.CD4.CXCR4 tumour cells were xenografted to BALB/c nu/nu athymic nude mice and when the tumour reached at least 80 mm³, the mice were injected with the tracer and scanned over 60 min (Figure 12 and Figure 13).

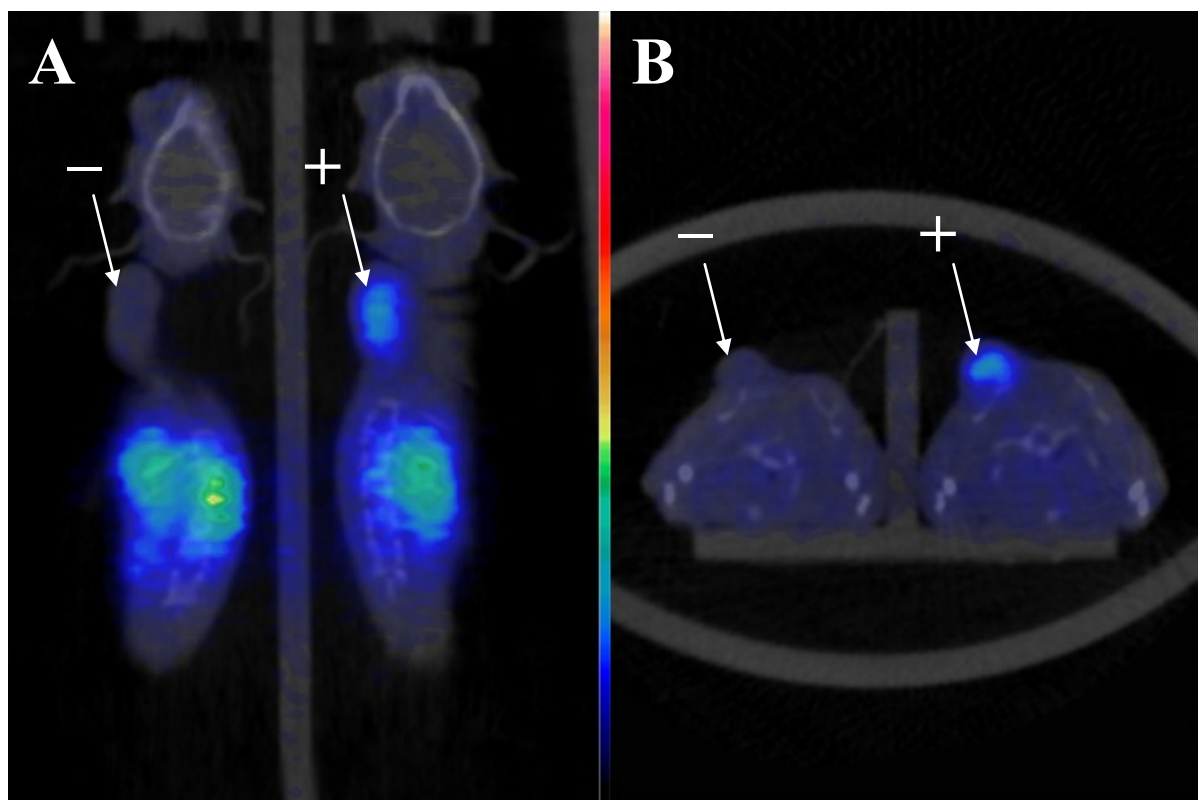


Figure 12. Dynamic CT/PET imaging of 3.7 MBq of [⁶⁸Ga]22 in mice bearing either U87.CD4- ("-") or U87.CD4.CXCR4- ("+") derived tumours ($n = 6$ and 5 , respectively). A: Coronal CT/PET images of representative animals. B: Axial CT/PET images of representative animals. White arrowheads indicate the tumours.

As seen in the imaging data, the difference of uptake between CXCR4-positive and -low cells lines can still be appreciated *in vivo*. Moreover, U87.CD4.CXCR4 tumours were readily distinguishable from the environing healthy tissue, suggesting accumulation of our tracer in a CXCR4-dependent fashion.

Defining regions of interest around the tumours in selected imaging frames allowed us to plot the concentration of radioactivity measured therein *versus* time. The resulting time *versus* activity curves yielded by dynamic PET imaging of U87.CD4 or U87.CD4.CXCR4 tumour-

bearing mice illustrated the difference in pharmacokinetics of [^{68}Ga]**22** between CXCR4-low and -positive tumours (Figure 13).

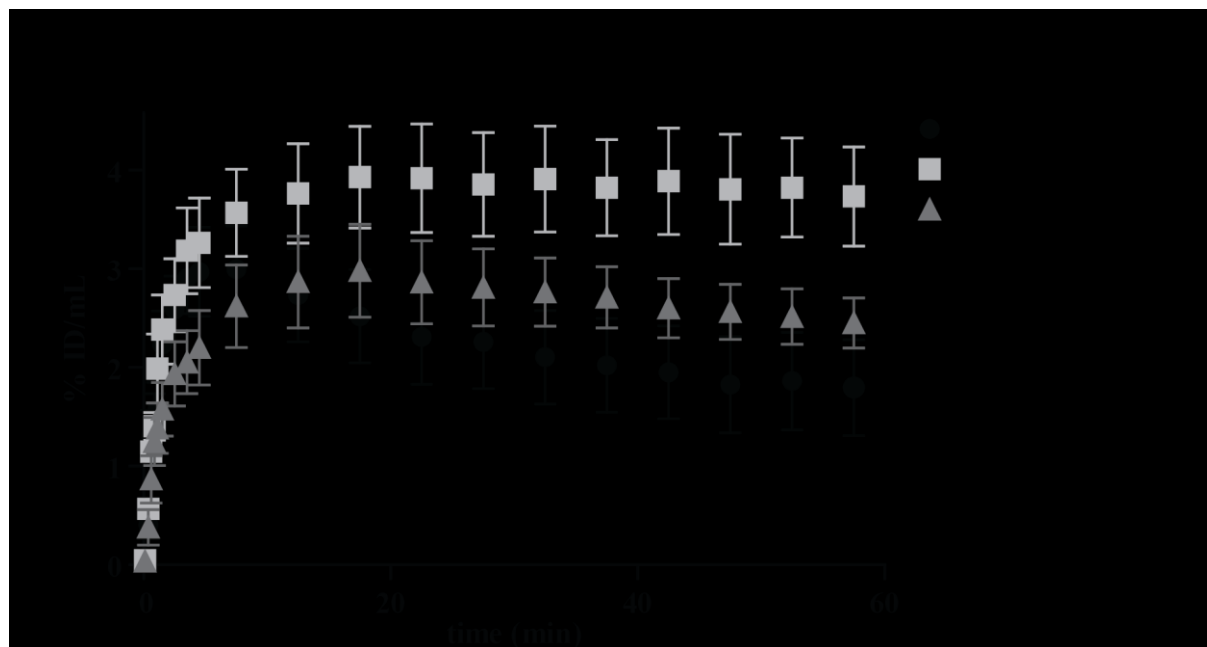


Figure 13. Tumour time *versus* activity curves for the dynamic CT/PET imaging of 3.7 MBq of [^{68}Ga]**22** in mice bearing either U87.CD4- or U87.CD4.CXCR4-derived tumours, including blockade with 50 μg of **22** ($n = 6, 5$, and 5 , respectively). Data are mean $\pm$ SEM.

In particular, the tracer accumulated steadily in U87.CD4.CXCR4 cells over 20 min before plateauing for the remainder of the scan; on the other hand, an increase of radioactivity was measured in U87.CD4 cells over 10 min followed by steady diminution. As a result, the normalised uptake value at 60 min was significantly higher in CXCR4-positive than in -low tumours (3.73% ID/mL and 1.80% ID/mL, respectively), suggesting *in vivo* potency of **22** as a radiotracer.

Co-injection of U87.CD4.CXCR4-bearing mice with 50 μg of **22** presented gradual tracer uptake in the tumour over 20 min followed by a decline similar to that observed in U87.CD4 tumours. Consequently, normalised uptake value at 60 min was partially reduced during the blocking study (2.45% ID/mL).

Specificity of the tracer for CXCR4 can be further assessed by analysing the tumour-to-muscle ratios (Figure 14).



Figure 14. Tumour-to-muscle ratio at 60 min for the dynamic CT/PET imaging of 3.7 MBq of [^{68}Ga]**22** in mice bearing either U87.CD4- or U87.CD4.CXCR4-derived tumours, including blockade with 50 μg of **22** ($n = 6, 5$, and 5 , respectively). Data are mean $\pm$ SEM and statistical significance was confirmed using a 1-way ANOVA with Bonferroni post-tests ($P < 0.05$).

Thus, CXCR4-low cells exhibited a tumour-to-muscle ratio of 1.27 while CXCR4-positive cells exhibited a ratio of 2.85. This latter uptake could be significantly blocked down to a value of 1.62 by co-injection with **22**.

Taken together, the data issued by these preliminary *in vivo* experiments showed that [^{68}Ga]**22** is a CXCR4-specific tracer offering efficient discrimination between CXCR4-expressing tumours and CXCR4-low cells (tumours or background tissue) *in vivo*. This encouraged us to carry on with the preclinical validation of this tracer by dynamically following the biodistribution of the tracer in a more quantitative way.

4.3. Biodistribution

In order to quantify the tracer accumulation in specific organs, tumour-bearing mice were sacrificed at the end of the imaging experiments (60 min post-injection), tissues were collected and their activity measured (Table 8 and Figure 15B).

Table 8. Tissue distribution of [^{68}Ga]**22** in U87.CD4- or U87.CD4.CXCR4-tumour bearing mice at 60 min post-injection, including blockade in U87.CD4.CXCR4-tumour bearing mice by co-injection of 50 μg of **22** ($n = 3, 4$ and 5 , respectively).

Tissue	Biodistribution of [^{68}Ga] 22 at 60 min (% ID/g)	
	No treatment	50 μg of [^{nat}Ga] 22
Blood	1.152 $\pm$ 0.200	1.452 $\pm$ 0.386
Plasma	1.467 $\pm$ 0.290	2.174 $\pm$ 0.441
Blood cells	0.617 $\pm$ 0.161	0.467 $\pm$ 0.164
Heart	0.817 $\pm$ 0.093	1.351 $\pm$ 0.267
Lung	3.104 $\pm$ 0.513	3.573 $\pm$ 0.755
Liver	21.034 $\pm$ 6.154	11.799 $\pm$ 5.584
Gallbladder	21.129 $\pm$ 6.221	11.552 $\pm$ 5.848
Stomach	0.977 $\pm$ 0.367	1.375 $\pm$ 0.359
Duodenum	1.037 $\pm$ 0.155	1.325 $\pm$ 0.290
Jejunum	0.840 $\pm$ 0.136	1.362 $\pm$ 0.238
Caecum	0.432 $\pm$ 0.075	0.689 $\pm$ 0.140
Colon	0.844 $\pm$ 0.139	1.447 $\pm$ 0.243
Spleen	3.462 $\pm$ 0.782	3.027 $\pm$ 0.731
Kidney	24.004 $\pm$ 4.920	45.212 $\pm$ 16.688
Muscle	0.619 $\pm$ 0.078	0.899 $\pm$ 0.187
Bone	2.518 $\pm$ 0.531	2.443 $\pm$ 0.386
Brain	0.093 $\pm$ 0.010	0.149 $\pm$ 0.063
Urine	30.667 $\pm$ 11.590	66.298 $\pm$ 51.603
U87.CD4 tumour	1.582 $\pm$ 0.228	–
U87.CD4.CXCR4 tumour	5.329 $\pm$ 1.836	4.356 $\pm$ 0.970
U87.CD4 Tumour/Blood	1.364 $\pm$ 0.227	–
U87.CD4.CXCR4 Tumour/Blood	6.304 $\pm$ 1.845	2.600 $\pm$ 0.087
U87.CD4 Tumour/Muscle	2.373 $\pm$ 0.337	–
U87.CD4.CXCR4 Tumour/Muscle	9.502 $\pm$ 1.709	4.839 $\pm$ 0.751
U87.CD4 Tumour/Plasma	1.175 $\pm$ 0.118	–
U87.CD4.CXCR4 Tumour/Plasma	4.571 $\pm$ 1.525	1.945 $\pm$ 0.259

Data are **mean** $\pm$ SEM.

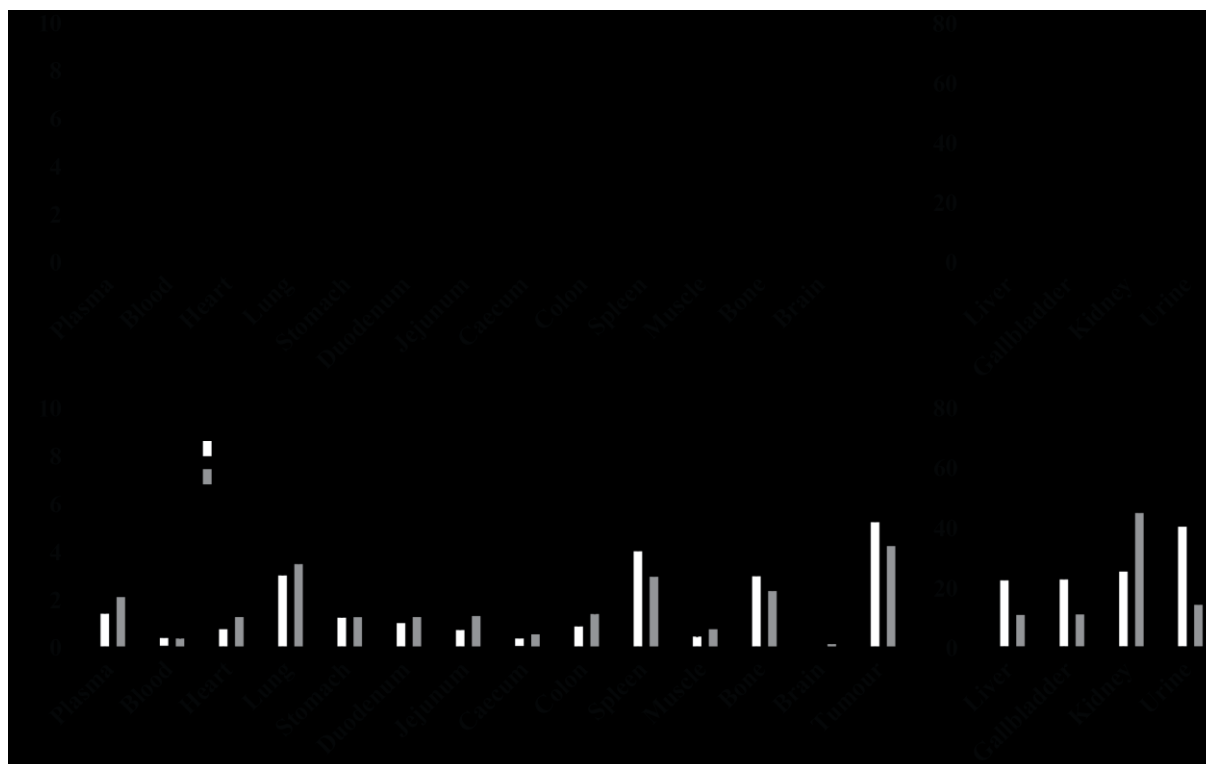


Figure 15. Tissue distribution of $[^{68}\text{Ga}]\mathbf{22}$, A: in non-tumour bearing mice at 2, 30, and 60 min post-injection ($n = 6, 4$ and 4 , respectively), B: in U87.CD4- or U87.CD4.CXCR4-tumour bearing mice at 60 min post-injection, including blockade in U87.CD4.CXCR4-tumour bearing mice by co-injection of $50\text{ }\mu\text{g}$ of $\mathbf{22}$ ($n = 3, 4$ and 5 , respectively). Data are mean $\pm$ SEM.

The most radioactive organs were the liver, gallbladder, kidney and urine (21, 21, 24 and 31% ID/g, respectively), suggesting hepatobiliary and urinary excretory routes, along with potential metabolic activity. In agreement with the PET data, uptake was found significantly higher in U87.CD4.CXCR4 than in U87.CD4 cells while co-injection of $\mathbf{22}$ with the tracer blocked the uptake of radioactivity in U87.CD4.CXCR4 tumours, although only to a limited extent (5.3, 1.6 and 4.4% ID/g, respectively). We believe that the blocking was weaker *in vivo* than *in vitro* because of the lower amounts of blocking agent used with the mouse, as well as the weight and volume of the mouse; also, co-injection instead of pre-injection of the blocking agent can result in lower blockade. On the other hand, co-injection of the blocking agent showed no decrease of radioactivity uptake in any other tissue, indicating that no specific binding occurred at unintended locations. More precisely, several tissues, in particular the kidneys and urine, presented increased accumulation of the tracer 60 min after the start of the blocking experiment (Table 8). This behaviour is typical of blocking experiments as blockade of the receptors increases tracer blood circulation and subsequent tracer excretion. While a

cell uptake of 5.3% ID/g could be observed in CXCR4-expressing tumours, an adequate tumour-to-muscle ratio of 9.5 was measured.

A more complete understanding of the pharmacokinetics of [^{68}Ga]**22** could be obtained by performing dynamic biodistribution experiments. For these experiments, healthy BALB/c mice were injected with the tracer and sacrificed at various time-points post-injection (2, 30 and 60 min). As earlier, tissues were harvested and their activity counted (Figure 15A). From these experiments, we could observe that the amount of radioactivity circulating in the plasma was constant through time, with negligible non-specific uptake by red blood cells. Moreover, radioactivity accumulation in certain tissues (liver, gallbladder, kidneys and urine) increased with time, giving further indication of excretion and possible metabolic activity. It is worth noting that at 60 min post-injection we obtained a liver-to-tumour ratio slightly lower than was reported in the literature for the other copper-64-labelled DO3A/NOTA derivatives at 4 h post-injection.¹⁰⁵ The rapid clearance observed with our tracer, along with the good discrimination between U87.CD4.CXCR4 cells and cells that do not over-express CXCR4 (U87.CD4 and background tissue), is deemed appropriate for a tracer as it reduces non-specific binding, therefore yielding a better contrast, and shortens the time of residence of the tracer in the body. Nonetheless, in order to progress the evaluation of our tracer, it was necessary to assess the tracer's metabolism rate and its bioavailability over time. Detailed study of the sources of radioactivity in metabolic tissues would allow us to have a better understanding of our tracer's pharmacokinetic properties.

4.4. Metabolite study

4.4.1. Brief description of the concept

Radioactive metabolic tissues can be harvested and prepared in order to identify the origin of the source of radioactivity by radio-HPLC. In our instance, we decided to monitor metabolic activity in liver, plasma and urine so as to be able to picture hepatic metabolism, tracer and metabolite blood circulation and urinary excretion, respectively.

However, the large amount of proteins found in biological samples requires preparation before using the HPLC. Thus, proteins need be precipitated with an organic solvent (*e.g.*, ice-cold MeOH or MeCN) and the samples centrifuged. The resulting supernatant is then formulated in a sample of HPLC mobile phase, filtered, and injected onto the HPLC column.

4.4.2. Optimisation of the sample preparation procedure

A few preliminary attempts at metabolite study were carried out. Liquid samples (plasma and urine) were precipitated with three volumes of ice-cold MeOH and the mixtures were vortexed and centrifuged. Unfortunately, in the case of plasma samples, most of the radioactivity was found in the pellet rather than in the supernatant, suggesting that strong interactions between our peptidic tracer and plasma proteins occur, which are detrimental to radioactivity recovery. In order to circumvent this issue, addition of an aqueous saturated solution of urea to the samples should allow further denaturation of the plasma proteins and release of our parent compound and metabolites. This was attempted using 100 μ L of urea solution with frozen plasma samples, using PBS as a control experiment (Figure 16).

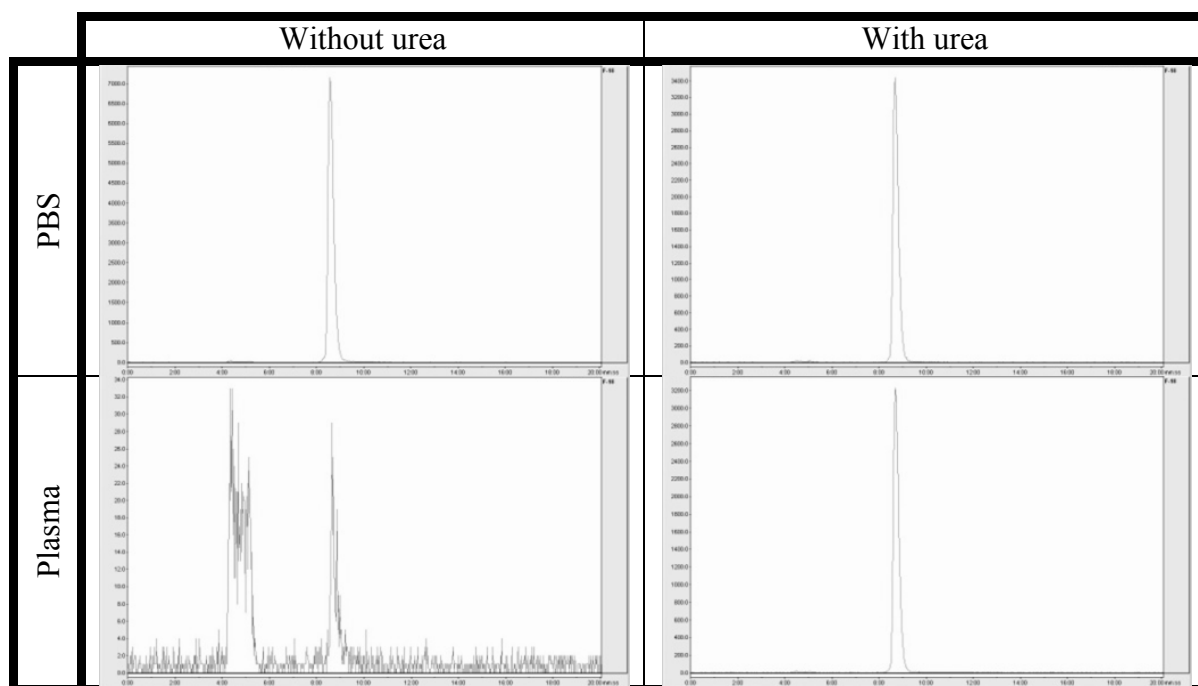


Figure 16. HPLC of the supernatant after sample preparation (PBS or frozen plasma) for metabolite study, with or without addition of urea.

It is clear that addition of urea allowed the release of the parent compound from frozen plasma sample. This was quantitatively assessed by measuring the recovery rate, defined as the ratio of the supernatant's activity to the total activity (supernatant + pellet). Without urea, only 1.3% of the activity was recovered. On the other hand, urea improved the recovery rate up to 81%. Unfortunately, the use of this method with fresh animal samples did not allow

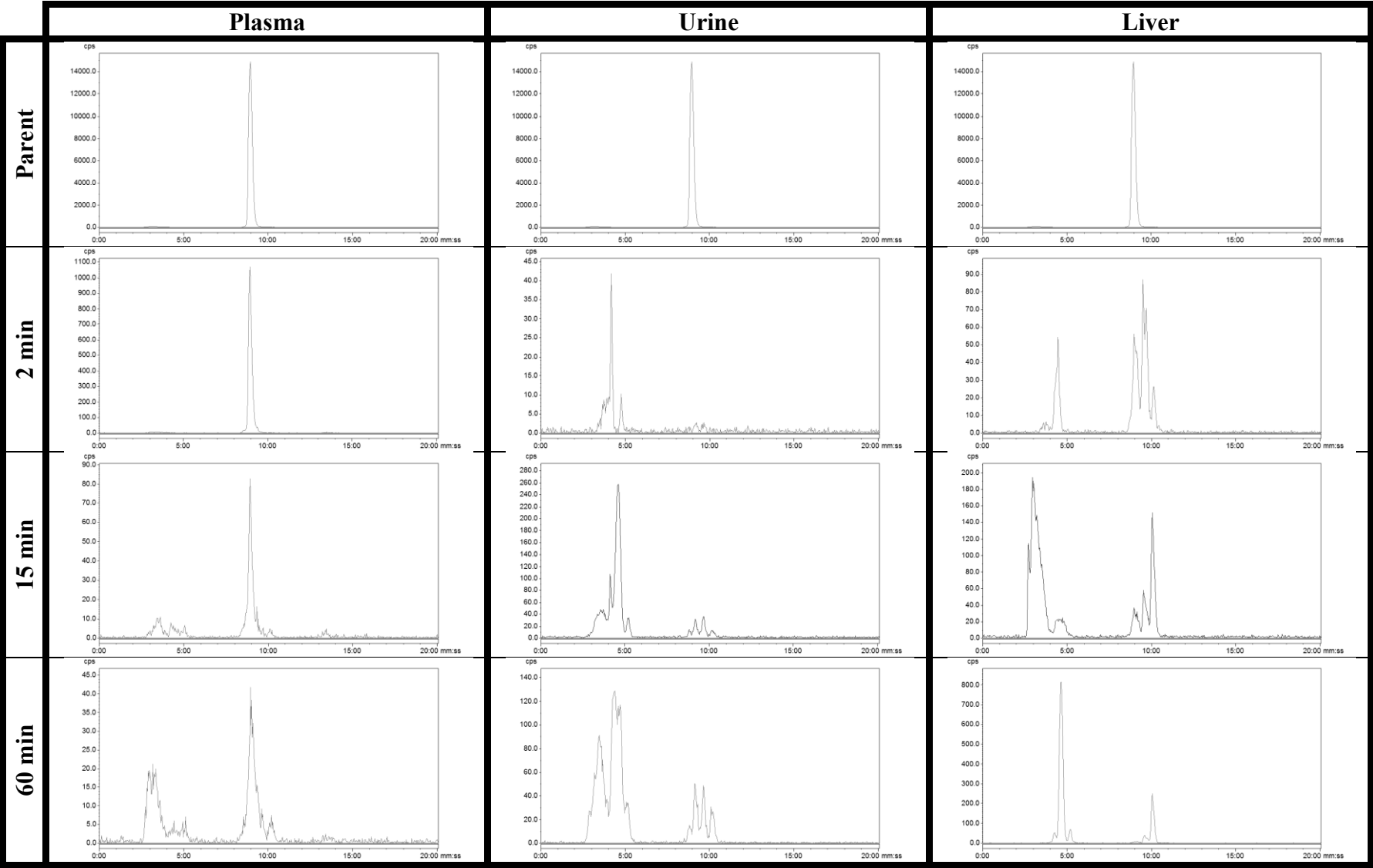
such an efficient recovery of the parent compound. Trying to circumvent this problem by modification of the HPLC characteristics (shorter run, higher flow rate, injection of more activity) showed only limited improvement.

Noticing that the frozen plasma samples were kept in a standard freezer ($-10\text{ }^{\circ}\text{C}$) while fresh plasma samples are frozen to $-78\text{ }^{\circ}\text{C}$ between harvest and preparation for metabolite study, we hypothesised that the freezing step might have had some influence on the recovery rate. Conserving the samples on wet rather than dry ice improved the recovery rate slightly. Finally, a step-wise precipitation of the liquid samples finished improving the recovery up to a suitable value. This was performed by adding a mixture of the biological sample and urea to an equivalent volume of ice-cold MeOH before vortexing, centrifuging and repeating the procedure with the resulting supernatant.

4.4.3. Results and discussion

Having developed a workable sample preparation method, it was possible to efficiently monitor the radioactive content of plasma, urine and liver at 2, 15 and 60 min post-injection (Table 9).

Table 9. Metabolite study of [⁶⁸Ga]22. Representative radiochromatograms for plasma, urine and liver preparations at 2, 15 and 60 min post-injection.



Comparison of the various HPLC chromatograms thus obtained to the radiotracer's provided a simple measure of metabolism. This can be quantified by measuring the amount of radioactivity corresponding to the parent compound as a ratio of the total activity (Table 10).

Table 10. Metabolite study of [^{68}Ga]22. Proportion of parent compounds in plasma, urine and liver samples at 2, 15 and 60 min post-injection.

	Plasma	Urine	Liver
2 min	94$\pm$7%	4.8$\pm$4.4%	4.7$\pm$1.7%
15 min	43$\pm$21%	5.6$\pm$1.0%	3.2$\pm$2.9%
60 min	33$\pm$12%	7.6$\pm$1.0%	5.1$\pm$3.6%

Proportion defined as $\frac{\text{AUC}_{\text{parent compound}}}{\text{AUC}_{\text{parent compound}} + \text{AUC}_{\text{metabolites}}}$. Data are **mean** $\pm$ SEM of three independent experiments.

Pleasingly, it was found that the tracer was present in good proportion in plasma, thus allowing continuous perfusion of the tumour with tracer. For instance, a third of the activity at 60 min post-injection corresponded to the tracer. This good blood circulation explains why the tumour's activity plateaued instead of decreasing over time (Figure 13), and confirms that our tracer is serum-stable for clinically relevant lengths of time.

Furthermore, we were delighted to see that nearly no parent compound was found in the urine, suggesting that no excretion of the tracer occurred. On the other hand, the urinary excretion route seemed to play in our favour by eliminating undesired metabolites from the body; these radiochemical species would for instance be able to make non-specific binding and therefore increase the noise during tomography.

Finally, nearly no tracer was found in the liver, evidence of efficient metabolism. Coupled with fast elimination of the resulting metabolites, such metabolism is desirable for a tracer so as to reduce the dose received by the patient over a scan.

5. Conclusions

A tracer for radiometal chelation based on the structure of TN14003, a potent tetradecapeptide derived from horseshoe crabs' defence mechanism has been designed. We aimed to improve several clinical aspects of the tracers published by Jacobson and co-workers,¹⁰⁵ in particular the long exposure of patient's liver and kidneys to copper-64's ionising radiations. This could be achieved with shorter-lived gallium-68 and size-reduction of the chelating agent bioconjugated to the peptidic core.

We chelated the designed precursor with various metals (gallium, aluminium fluoride, indium and copper) and their binding affinity was confirmed by *in vitro* radioligand competitive binding assay. This precursor was also the subject of a thorough screening of conditions, which allowed furthering our understanding of the concomitant formation of gallium colloid. Based on this knowledge, a reliable, fast and efficient labelling method for gallium-68 was devised, and was successfully employed in another part of this project (see section IV.4.1) but also in different hands for the radiosynthesis of various other tracers [such as a vascular endothelial growth factor receptor 2 (VEGFR-2)-targeting peptide or CXCR4-targeting AMD3465 small molecule]. Precursor **21** was therefore labelled in good EOS-RCY, excellent purity and high specific activity in approximately 11 min from elution to delivery.

In vitro cellular uptake assay, *in vivo* biodistribution and corresponding blocking studies confirmed potency and specificity of the tracer for CXCR4, with neither non-specific nor off-target binding. Imaging allowed visualisation of CXCR4-expressing tumours with appropriate contrast. Moreover, liver uptake was reduced compared to previous published work,¹⁰⁵ thus reducing the dose received by potential patients. Finally, metabolite study confirmed suitable blood circulation, fast tumour targeting and rapid excretion of metabolites. This should permit to scan just after tracer injection, releasing some of the patient's burden associated with long scanning protocols.

The results from the full validation of our tracer encourage us to carry on with clinical study with confidence. We hope that [⁶⁸Ga]**22** will allow visualisation of numerous metastatic cancers and quantification of their levels of CXCR4-expression. Moreover, patient stratification with gallium-68-labelled **21** could lead to personalised targeted radiotherapy with yttrium-90- or lutetium-177-chelates of the same precursor.

CHAPTER III.

CYCLOPENTAPEPTIDES

1. Design

1.1. Downsizing T140

FC131, or *cyclo*(–Gly1–D-Tyr2–Arg3–Arg4–Nal5–), is a cyclopentapeptide that was first synthesised by Fujii *et al.*⁴⁹ in 2003 (Figure 17).

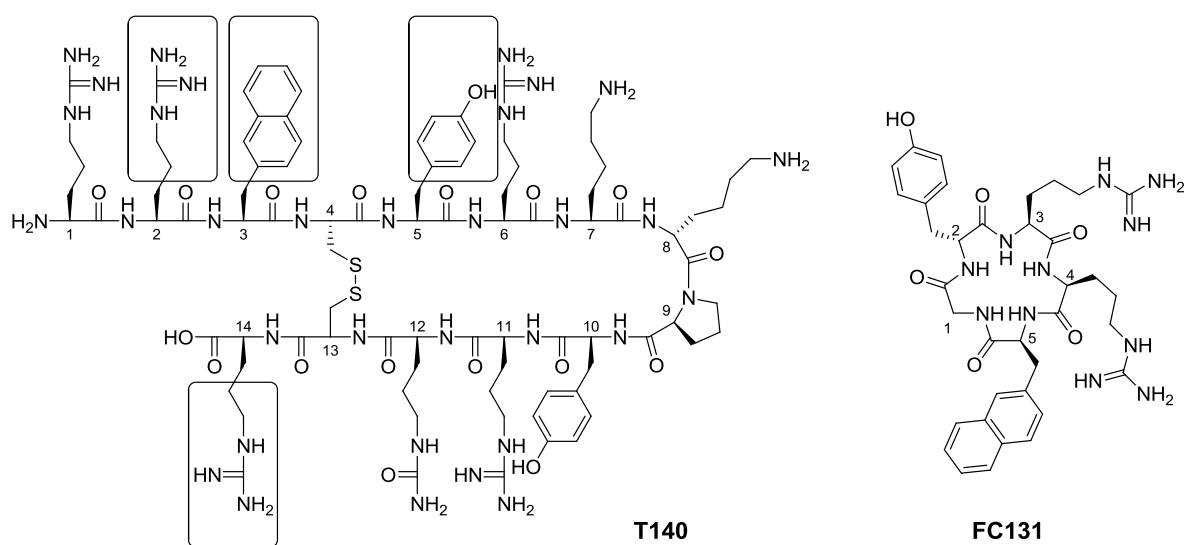


Figure 17. Structures of T140 and FC131. The four crucial residues in T140 are boxed.

The rationale behind its design came from the detailed study of the indispensable residues of T140 for its intrinsic bioactivity against HIV entry (see section II.1). Thus, performing a standard alanine scan, *i.e.* the systematic replacement of one of T140's residue for D- or L-alanine in order to measure the cruciality of the replaced residue, Fujii *et al.* found that only four residues were of major importance: Arg2, Nal3, Tyr5 and Arg14.¹²⁹ Because of the

spatial proximity of these four residues in T140, the authors envisaged the possibility that a combination of these four residues along with glycine could prove equipotent to T140.⁴⁹

A library of cyclopentapeptides of various sequences and configurations was synthesised and their activities were assessed against CXCR4 in a competitive radioligand binding assay. The most potent of them, FC131, eventually presented an affinity for CXCR4 comparable to that of T140. It is not until 2011 that FC131 was used as a basis for the design of CXCR4-specific PET tracers, using gallium-68.¹³⁰ More recently, and based on the following original SAR analysis (see section II.1.2), our group published the radiosynthesis and preliminary biological evaluation of the CXCR4-specific probe labelled with *para*-[¹⁸F]fluorobenzaldehyde, tracer [¹⁸F]6, which showed extremely fast clearance.¹⁰²

1.2. Structure-activity relationship analysis

Analysis of the binding potencies of over 120 FC131 analogues available in the literature¹³¹⁻¹³⁸ provided valuable insight into SAR of these compounds (Figure 18).

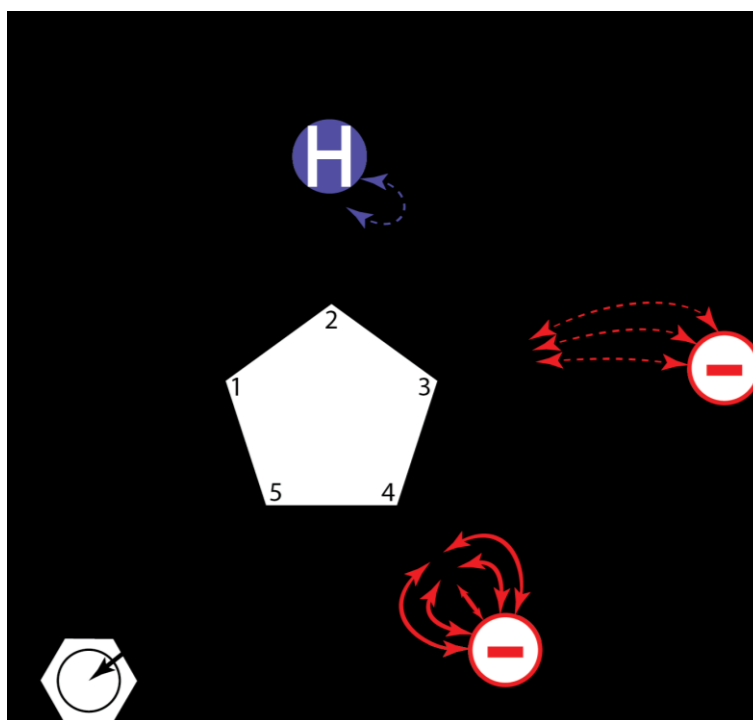


Figure 18. Schematic representation of CXCR4's binding site (black curved line) as described by the SAR analysis. FC131 is represented as a pentagone. Salt bridges are depicted in red, hydrogen bonds in blue, hydrophobic contact in black.

Substitution of Gly1 for another residue tends to cause significant loss of activity.^{131,132} This is probably due to a resulting change in the peptide's backbone conformation, as this residue is important to shape the cyclopentapeptide in a type II' β -turn, mimicking that of T140.^{49,139} Furthermore, Gly1 is required during the synthesis of such cyclopentapeptide to avoid racemisation concomitant with the cyclisation of linear pentapeptides.

On the other hand, D-Tyr2 has been successfully substituted for a variety of small aromatic residues (*e.g.*, D-Phe, D-Phe(2-F), D-Phe(3-F) and D-thienylalanine) or even larger aromatic residues provided that they do not extend in its C $^{\zeta}$ -direction (*e.g.*, 1-naphthylalanine). Failing that, a drop in the binding affinity could be measured (*e.g.*, the series D-Phe(4-NH₂), D-Phe(4-F), D-Phe(4-OMe), D-Phe(4-Cl), D-Phe(4-Br)).^{132,133,138} All this suggests a potential steric hindrance on this position but also the presence of a stabilising hydrogen bonding. Therefore, it might be anticipated that the substitution of this residue for other small aromatic residues, such as a triazole-containing one, could be accepted.

Substitution of Arg3 seems to have a relatively moderate effect on the binding as long as the replacing residue is not negatively charged such as glutamate.^{131,132,135,138} We therefore expected this particular position to form a weak salt bridge with a distant negatively charged residue in a large pocket, making the 3-position attractive for modification.¹³⁰

Conversely, any modification of Arg4 (*e.g.*, 2,4-diaminobutyric acid, ornithine, lysine, guanidinolysine, histidine, and glutamate) was unsuccessful,^{132,138} entailing a strong electrostatic interaction with a negatively charged residue.

Finally, few instances of successful substitution of Nal5 for other fused bicyclic aromatic rings have been reported (*e.g.*, L- and D-benzothiaz-2-ylalanine and Trp)^{131,138} but as for D-Tyr2 it can be expected that its replacement for a small aromatic group could be satisfactory.

Although this data is important to rationally design candidate tracers based on FC131, we considered that the tracers' conception would benefit from stronger evidence of the interactions between FC131 and CXCR4.

1.3. Docking studies

As mentioned previously, the co-crystal structures of CXCR4 and antagonists were published in 2010.¹² In particular, CXCR4 was co-crystallised with CVX15, a 16-mer peptide close in structure to T140 (CVX15 = [Cit6, D-Lys8]T140–Gly–D-Pro–OH). Because of the structural

proximity of CVX15 to T140, and because FC131's structure is derived from that of T140, we decided to use this crystal structure to carry out *in silico* work in order to deepen our understanding of the nature of the binding of FC131 to CXCR4.

The following work was carried out with the Schrödinger software package.¹⁴⁰⁻¹⁴⁴ CXCR4 and CVX15 crystallised as homodimers (PDB: 3OE0),¹² and therefore the coordinates of one of the monomers were removed from the crystal structure. The receptor and ligand were treated with ProteinPrep using default settings (correction of crystal structure incongruities, assignment of bond orders and charges, treatment of disulfide bridges) and the resulting structure was minimised in the default forcefield (OPLS2005). Finally, a docking grid was then created around CVX15.

Minimisation of the three-dimensional structure of FC131 afforded 197 conformations with the default settings. The three conformations of lower energy were docked in the grid with Glide, affording 45 poses in total. These 45 poses were presenting comparable docking scores as 34 of them showed a docking score ranging between 1.0 and 0.8 time the lowest docking score (−9.00). The manual comparison of the 34 poses resulted in the following observations.

Nineteen poses were superimposing very well with each other, with Arg4 and Nal5 presenting the same interactions with CXCR4 in all poses, and D-Tyr2 and Arg3 presenting the same interactions with CXCR4 in 17 poses (Figure 19).

In more detail, the second aromatic ring of Nal5 was found inserted between Tyr116 and Arg188 with which it makes van der Waals and π -cation interactions, respectively. It can also be seen clearly from the ligand interaction diagram that this same ring interacts with His203 by π -stacking. Arg4 makes two salt bridges with Asp97 and Asp187, while Arg3 makes only one with Glu32, being exposed to solvent. Similarly, D-Tyr2 is stabilised by the formation of a hydrogen bond with Tyr190 and by multiple hydrophobic contacts with CXCR4. Meanwhile, it is being exposed to the solvent to some extent. Finally, Gly1 does not seem to produce any interactions with CXCR4.

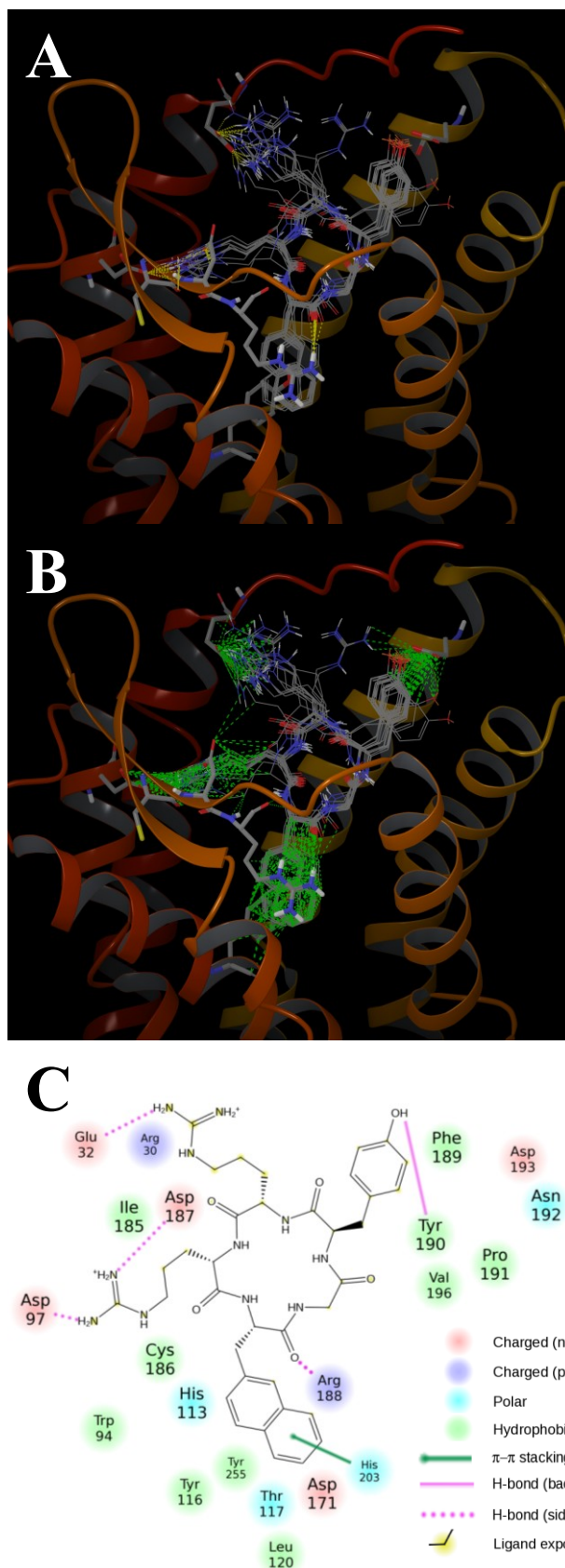


Figure 19. Overlay of 19 poses of FC131 docked in CXCR4. A: Depiction of intermolecular hydrogen bonds. B: Depiction of the "good" van der Waals intermolecular contacts (as defined by default settings). C: Ligand interaction diagram.

These results are in accordance with the observations made from the SAR. Consequently, we can consider our docking approach validated and conclude with confidence that out of the 5 amino acids present in FC131, Gly1 and Arg4 are the less likely to accept modifications. The poor substitutability of Gly1 cannot be accounted for by any interactions with CXCR4 and should therefore be explained by its importance in shaping the cyclopentapeptidic backbone. On the other hand, Arg4's strong interactions with CXCR4 make this amino acid crucial for affinity. Nal5 interacts deep within CXCR4's binding pocket; its modification for a small aromatic ring, such as a triazole-containing non-proteogenic amino acid, could allow retention of affinity for CXCR4, provided the CXCR4-ligand complex is flexible enough to allow the same π -stacking interactions as witnessed in the case of Nal5. In the same way, it is anticipated that D-Tyr2's steric hindrance and hydrophobic contact could be used to introduce a small aromatic substitution. Its greater solvent exposure makes it a better candidate for modification. Finally, Arg3's electrostatic interactions are confirmed, but also its steric capacity, pointing towards the extracellular matrix.

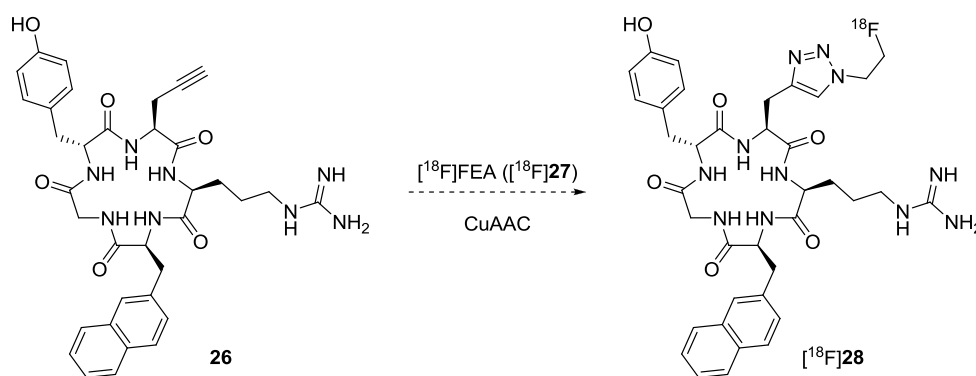
To summarise, the suitability of FC131's residues for substitution in order to allow labelling can be ranked as follows: Arg3 > D-Tyr2 > Nal5 > Arg4.

1.4. Choice of labelling strategy

A variety of labelling strategies and isotopes are available to the radiochemist (see section I.5). We selected fluorine-18 because its relatively long half-life (~110 min) and superior positron emission characteristics (97% of β^+ disintegrations) designate it as the isotope of choice for PET imaging. Gallium-68, as depicted by our work on the tetradcapeptide tracer described previously (see chapter II), requires a large chelating group that might not be accommodated easily by the tracer. The use of such a large pendant group was made possible by the size of **22**, which likely protrudes out of CXCR4's helix bundle when bound to it.

The possible acceptance of small aromatic residues at FC131's 2- and 5-positions pointed towards the selection of the click labelling strategy. Indeed, the resulting triazole is slightly smaller than the benzyl and the naphthyl aromatic rings found in D-Tyr and Nal, thus potentially allowing them to fit the corresponding binding pockets. Moreover, *N*-fluorenylmethyloxycarbonyl (Fmoc) -protected propargylglycine, abbreviated Fmoc-Prg-OH thereafter, is commercially available and is amenable for solid-phase peptide synthesis.

In order to confirm the assumptions derived from our two orthogonal analyses of the CXCR4 binding pocket, namely SAR and docking studies, we decided to start our quest for an FC131-based tracer by the substitution of Arg3 to allow radiolabelling by click chemistry (Scheme 12).



Scheme 12. Proposed radiolabelling of precursor **26**, an Arg3-substituted analogue of FC131.

Thus, precursor **26** would be made available by solid-phase peptide synthesis, and its radiolabelling with 2- ^{18}F fluoroethyl azide (^{18}F FEA, ^{18}F **27**) was expected to afford the desired tracer ^{18}F **28**.

2. Labelling at the 3-position

2.1. Reference compound and precursor

2.1.1. Solid-phase peptide synthesis strategy

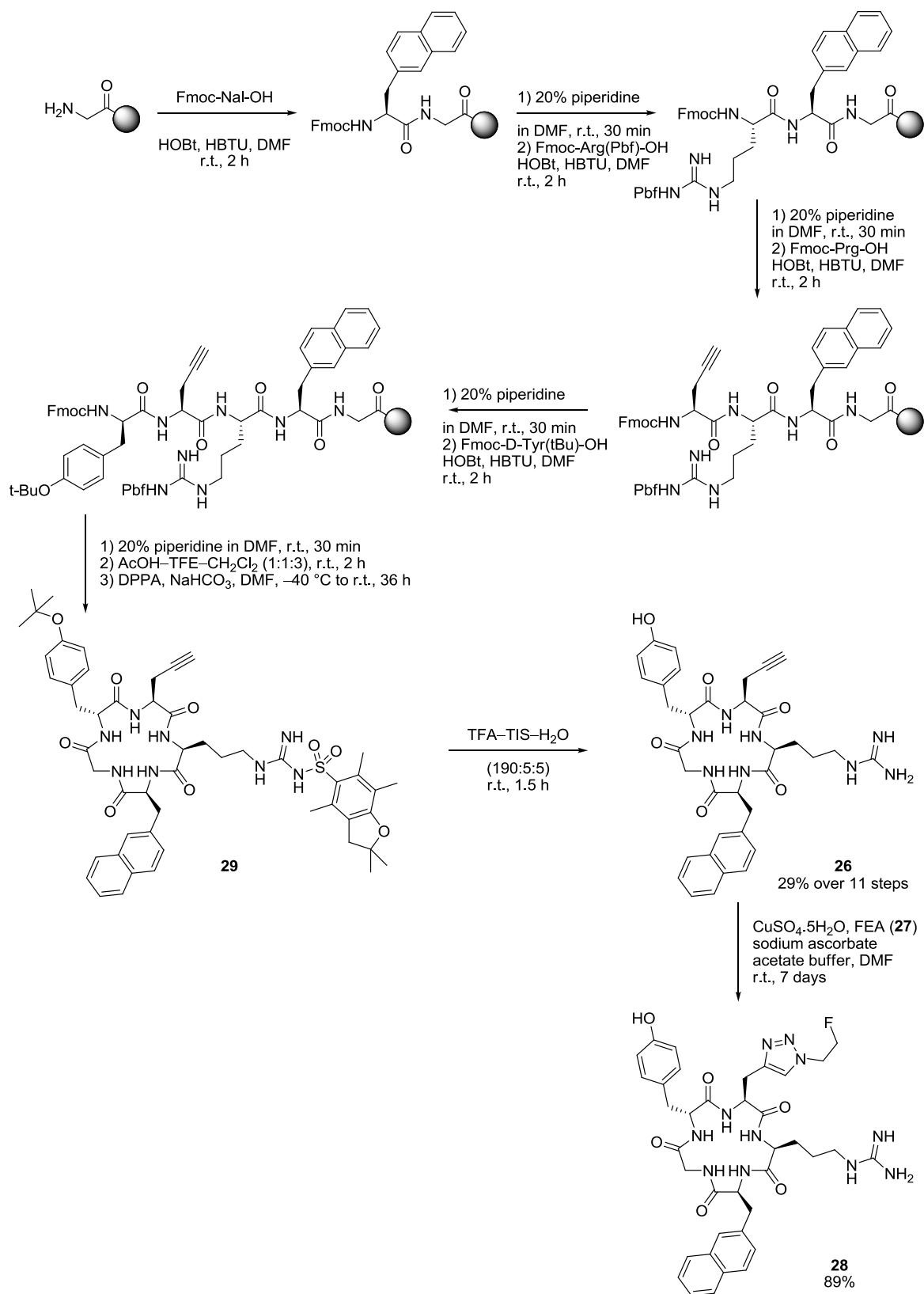
Because automated peptide synthesisers use a large excess of reagents and our pentapeptide features three unnatural and therefore expensive amino acids, we decided to carry out the synthesis manually. Furthermore, it was considered as necessary to be able to cleave the linear pentapeptide from the resin without removal of the protecting groups of the residues' side chains. This would prevent various functional groups interfering with the subsequent cyclisation step. To this end, we chose to use a 2-chlorotrityl resin as it allows cleavage of the resin under relatively mild acidic conditions (AcOH – trifluoroethanol (TFE) – CH_2Cl_2 1:1:3), while the usual side chain protecting groups employed for Fmoc-strategy (tBu for Tyr, Pbf for

Arg) need harsher conditions to be cleaved (typically, TFA – triisopropylsilane (TIS) – H₂O 190:5:5).

2.1.2. Synthesis

We opted to deploy a 2-chlorotrityl / Fmoc strategy (Scheme 13).

A mixture of hydroxybenzotriazole (HOBt), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), ethyldiisopropylamine (DIPEA) and *N*-Fmoc-protected amino acid (3 equivalents to 1 of resin bound free amine) was used to perform the amide bond formation while Fmoc-protecting groups were removed with 20% piperidine in DMF. Repeating successive amide bond formation and Fmoc-deprotection afforded a linear resin-bound pentapeptide, which could be cleaved from the resin upon treatment with an AcOH–TFE–CH₂Cl₂ (1:1:3) mixture. Cyclisation with diphenylphosphorylazide (DPPA) in high dilution went smoothly to yield the protected cyclopentapeptide **29**. Side chain protecting groups (tBu and Pbf) were readily removed upon shaking in TFA–TIS–H₂O (190:5:5) and the resulting peptide was precipitated with Et₂O and purified by preparative HPLC, thus affording the desired alkyne-containing precursor **26** in 29% yield from the commercially available Fmoc-Gly-2ClTrt resin. This peptide was carried forward by undergoing Huisgen alkyne-azide cycloaddition with FEA (**27**) mediated by copper(II)/sodium ascorbate solution. Preparative HPLC purification efficiently afforded the desired reference compound **28** in 89% isolated yield.

Scheme 13. Solid-phase peptide synthesis of precursor **26** and reference compound **28**.

2.1.3. Binding affinity

Binding activity of the TFA salt of compound **28** was assessed by competitive displacement of [^{125} I]SDF-1 α on U87.CD4.CXCR4 cells (Figure 20).

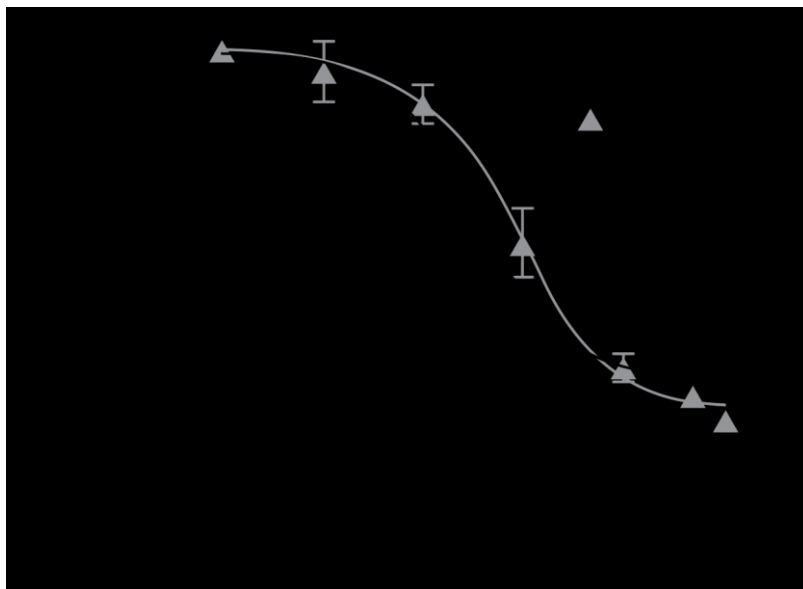


Figure 20. Radioligand binding assay comparing the affinities of FC131 and **28** to CXCR4. Data are mean $\pm$ SEM of three independent experiments.

The concentration of compound **28** that reduced the amount of radioactivity bound to the cells by 50% compared to control was determined after 60 min of incubation over ice. It showed an IC₅₀ value of 1.04 μ M, which is, to our delight, relatively close to what we found for FC131 (0.65 μ M).

This promising preliminary biological evaluation encouraged us to perform the radiosynthesis of [^{18} F]**28** in order to validate its properties as a CXCR4-specific tracer.

2.2. Development of the radiosynthesis

2.2.1. Synthesis of 2-[^{18} F]fluoroethyl azide

[^{18} F]FEA was synthesised following the procedure published by Glaser and Årstad⁷⁶ on a GE FASTlabTM automated platform. The native FDG cassette was modified to allow for the

drying of [^{18}F]KF and kryptofix, its reaction with 2-azidoethyl tosylate and the purification by distillation of the thus formed [^{18}F]FEA (Figure 21).

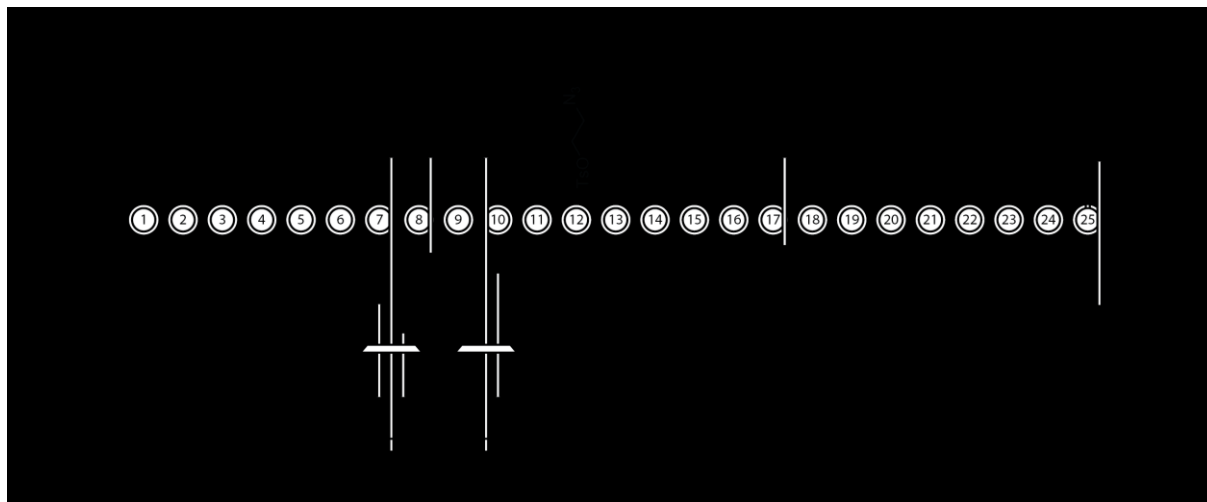


Figure 21. GE FASTlabTM cassette for the synthesis and purification of [^{18}F]FEA.

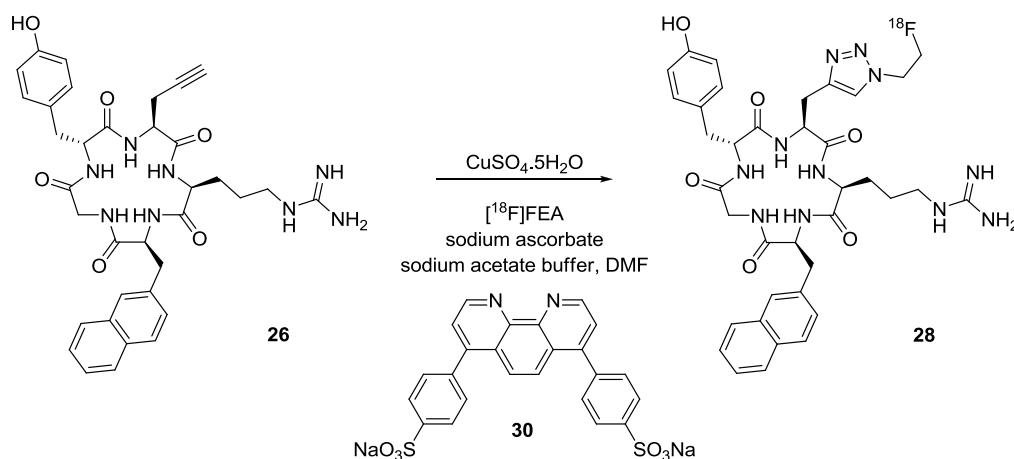
Briefly, the cassette is formed around a main backbone tubing with 25 3-way valves in series controlled by the automated platform and allowing the diversion of liquid mixtures with the assistance of N_2 pressure and vacuum. Some valves are connected to reservoirs or syringes while others can be connected to tubing. A heating block is also built into the platform.

Aqueous [^{18}F]fluoride is first trapped on a Waters QMA cartridge for preliminary drying and then eluted with a mixture of $\text{K}_{2.2.2}$ and KHCO_3 into the reaction vial. [^{18}F]KF is dried at $120\text{ }^\circ\text{C}$ with a flow of N_2 bubbling through and then 2-azidoethyl tosylate is added to the reaction mixture to form [^{18}F]FEA at $80\text{ }^\circ\text{C}$ in 15 min. Finally, the product is distilled into the collection vial (*via* the backbone tubing) to afford the prosthetic group in excellent radiochemical purity ($>99\%$) in $\sim 300\text{ }\mu\text{L}$ of MeCN. The total process proceeds in an overall $47\pm 7\%$ DC-RCY in $\sim 40\text{ min}$ ($n = 6$).

2.2.2. Optimisation of the labelling step

Previously, our group published an efficient radiosynthesis of [Tyr^3]octreotate analogues with [^{18}F]FEA.¹⁴⁵ In this procedure, $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ (2.0 equiv.), sodium ascorbate (2.2 equiv.), accelerating ligand bathophenanthroline disulfonic acid disodium¹⁴⁶ (**30**) (2.5 equiv.),

[^{18}F]FEA and the precursor (1.5 μmol) are stirred at room temperature for ~5 min before being purified by semi-preparative HPLC and reformulated by SPE (Scheme 14).



Scheme 14. Radiolabelling of **26**.

We decided to use the published labelling procedure as a starting point for the radiolabelling of precursor **26** (Figure 22).

Although after 3 min the conversion was measured as being 64% by radio-HPLC, it increased to 95% at 10 min ([precursor] = 10.9 mM). Attempts at reducing the amount of precursor showed that the reaction rate was steady when concentrations of 5.4 and 2.2 mM of precursor were employed, but suddenly dropped when using a 1.1 mM solution of precursor. This type of "threshold" behaviour for copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) has been established in the literature.¹⁴⁷ We were pleased to observe the quasi-completion of the reaction at 10 min and wondered whether improvement was possible by increasing the reaction temperature (Figure 23).

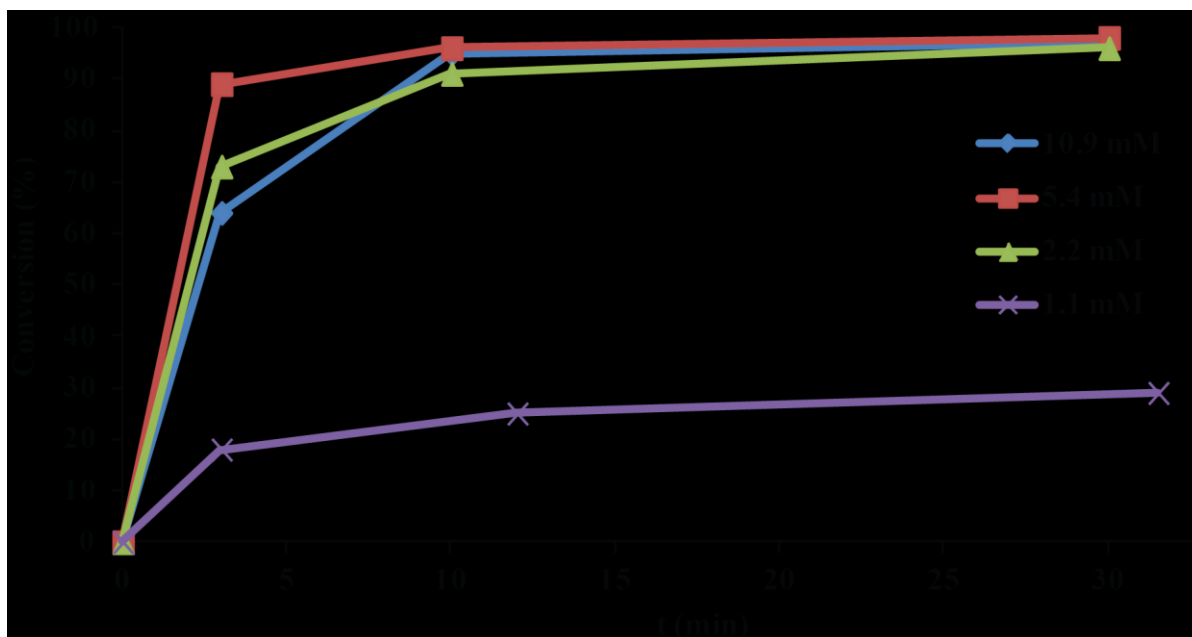


Figure 22. Advancement of the labelling followed over time at various peptide concentrations. $[^{18}\text{F}]\text{FEA}$ was added to the reaction mixture at $t = 0$. Reaction advancement was calculated from the radio-HPLC chromatogram as the ratio $\frac{\text{AUC}_{\text{product}}}{\text{AUC}_{\text{FEA}} + \text{AUC}_{\text{product}}}$.

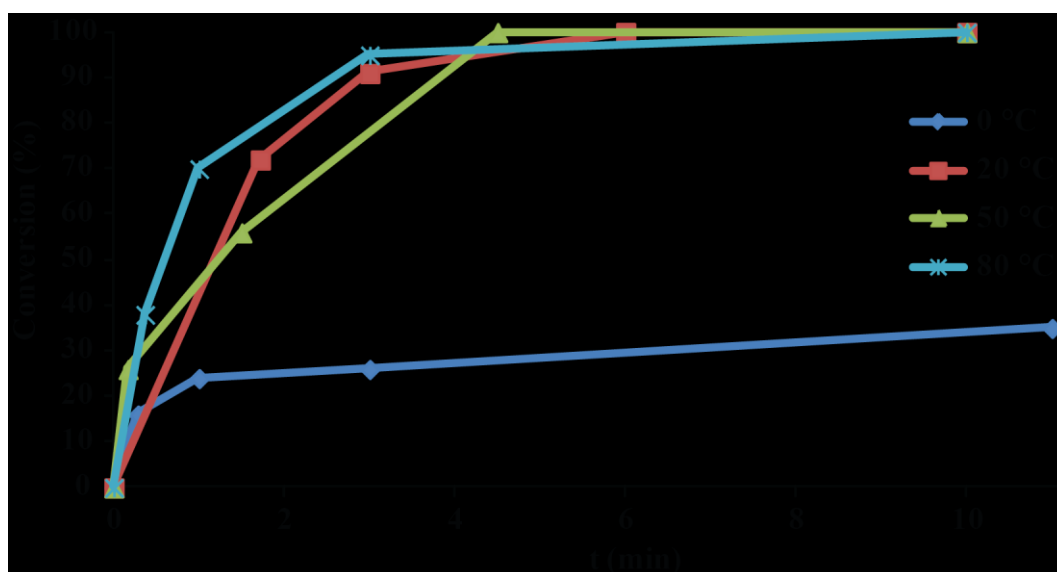


Figure 23. Advancement of the labelling followed over time at various reaction temperatures. $[^{18}\text{F}]\text{FEA}$ was added to the reaction mixture at $t = 0$. Reaction advancement was calculated from the radio-HPLC chromatogram as the ratio $\frac{\text{AUC}_{\text{product}}}{\text{AUC}_{\text{FEA}} + \text{AUC}_{\text{product}}}$.

Although increasing the temperature to 50 °C and 80 °C did not seem to improve the reaction rate, cooling the reaction to 0 °C allowed the detection of another "threshold" effect as seen before. Meticulous analysis of the radiotracer's peak showed a deformation of the peak at higher temperature, which was not observed at room temperature (Figure 24).



Figure 24. Comparison of the shape of the tracer's radiopeak for reactions carried out at different temperatures.

Tracer [^{18}F]**28**'s radiopeak became broader at increased temperatures, and a second peak appeared at the left hand side of the main peak for reactions carried out at 80 °C. We attributed this behaviour to the deterioration of the peptide in presence of Lewis acidic copper(I) by epimerisation of tracer [^{18}F]**28**'s stereogenic centres or chemical modification of the peptide. Although ligand **30** is designed to stabilise copper and to reduce its propensity to decompose organic material,¹⁴⁷ it is possible that higher temperatures favour the departure of the ligand thus leading to undesired by-products.

As a result, carrying out the labelling reaction at room temperature seemed more prudent, along with a peptide concentration of 5.4 mM. Thus performed, the radiolabelling reaction proceeded smoothly to full conversion in 10 min. Nevertheless, the large quantity of precursor needed (0.75 nmol, 0.59 mg) combined with our inability to separate the unreacted precursor from the tracer by semi-preparative HPLC resulted in poor apparent specific activity, typically $0.21 \text{ GBq} \cdot \mu\text{mol}^{-1}$. A poor specific activity negatively impacts the biological results, as explained earlier (see section I.3.4). As it was not possible to reduce significantly the peptide concentration of the reaction mixture in order to improve the specific activity of the delivered material, we devised a method to circumvent this issue by means of chemical scavenging of the unreacted precursor.

2.3. Chemical scavenging as a means of improving specific activity

2.3.1. Scavenging strategies

Ideally, the scavenging reaction needs to be fast and to effect a significant modification of the physico-chemical properties of the unreacted precursor **26** so as to allow separation of the scavenged peptide and tracer $[^{18}\text{F}]\textbf{28}$. Because the only difference between the tracer and the precursor resides on the 3-position in the form of a triazole and of an alkyne, respectively, it was natural to aim at using the acetylenic moiety for scavenging of the unreacted precursor. Furthermore, as the crude reaction mixture contains copper(I) and ligand **30**, it is ready to react with a scavenging azide, thus providing opportunity for a practical, rapid, one-pot labelling–scavenging procedure.

Commercially available or readily synthesisable azides are numerous, thus allowing diversity in the scavenging's *modus operandi*. It is for instance possible to use a lipophilic azide, a perfluorinated azide or a resin-bound azide. Adding a lipophilic azide in the reaction mixture at the end of the labelling step would allow consumption of unreacted precursor **26** and formation of a scavenged peptide that would be strongly retained on the semi-preparative HPLC column. This would permit isolation of tracer $[^{18}\text{F}]\textbf{28}$ with only the minimal amount of the unreacted precursor **26** that would have evaded scavenging (Figure 25).

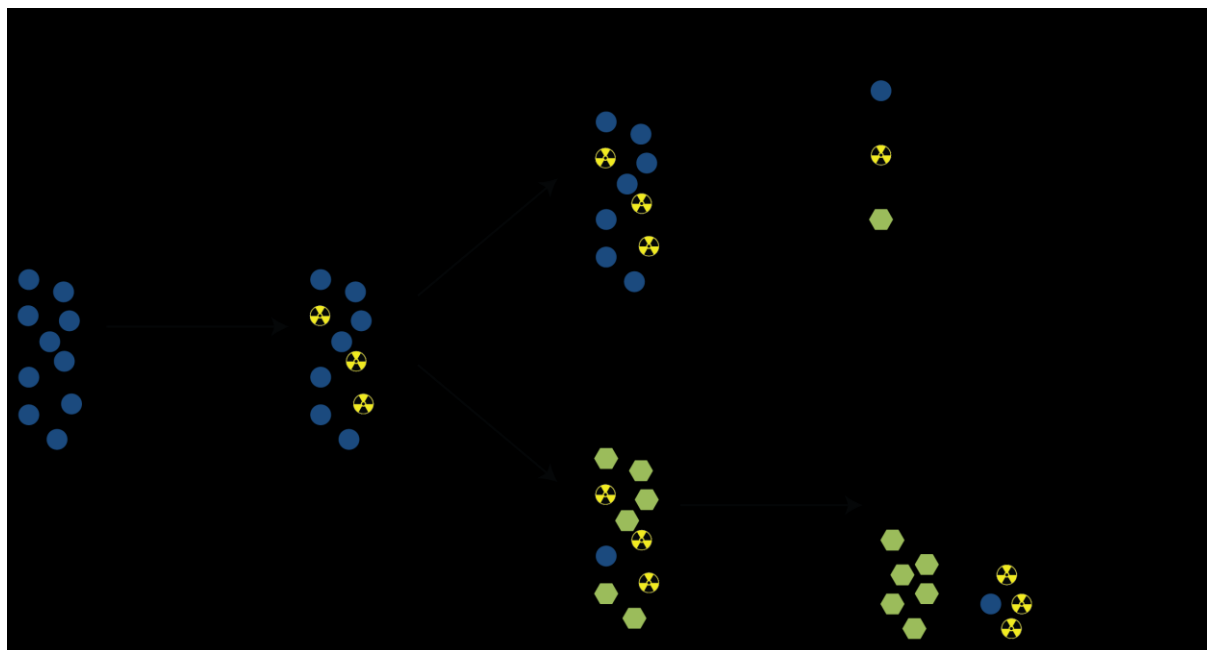


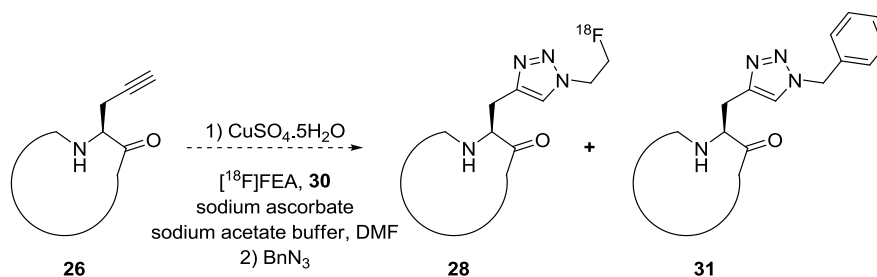
Figure 25. Schematic representation of the scavenging process with a lipophilic azide.

Similarly, it should be possible to add a perfluorinated azide in order to scavenge the unreacted precursor. The resulting scavenged peptide could be trapped on a fluorous SPE cartridge, yielding a mixture of hydrophilic compounds, the tracer and the ligand that would still need to be purified. It might even be possible to purify this mixture by C₁₈ SPE, thus affording an HPLC-free procedure for delivery of radiotracer. Finally, the use of a resin-bound azide should allow similar strategy with the use of a mere filtration instead of the fluorous SPE.

The lipophilic azide approach seems simpler as it only requires optimisation of the scavenging reaction. We therefore decided to investigate this strategy first.

2.3.2. *Lipophilic scavenging*¹¹⁷

Benzyl azide¹⁴⁸ was chosen as a potential scavenging agent (Scheme 15).



Scheme 15. Proposed labelling–scavenging sequence.

A test reaction demonstrated that this azide was fit for purpose, leading to a satisfactory 2-min difference in retention times between the radioactive tracer [^{18}F]**28** and the benzyl triazole derivative **31** (Figure 26).

Thus, within 10 min of scavenging, nearly no UV absorbance was observed at the retention time of the tracer. On the other hand, a new UV signal at 13.7 min appeared, corresponding to the scavenged species.

More detailed monitoring of the scavenging reaction was performed again using UV spectroscopy to quantify the concentration of the relevant species (Figure 27).



Figure 26. Representative chromatograms showing the evolution over time of the concentration of tracer and precursor, scavenged peptide and BnN₃. A: radiotracer. B: t = 0 (just before the addition of BnN₃). C: t = 10 min. D: t = 20 min.

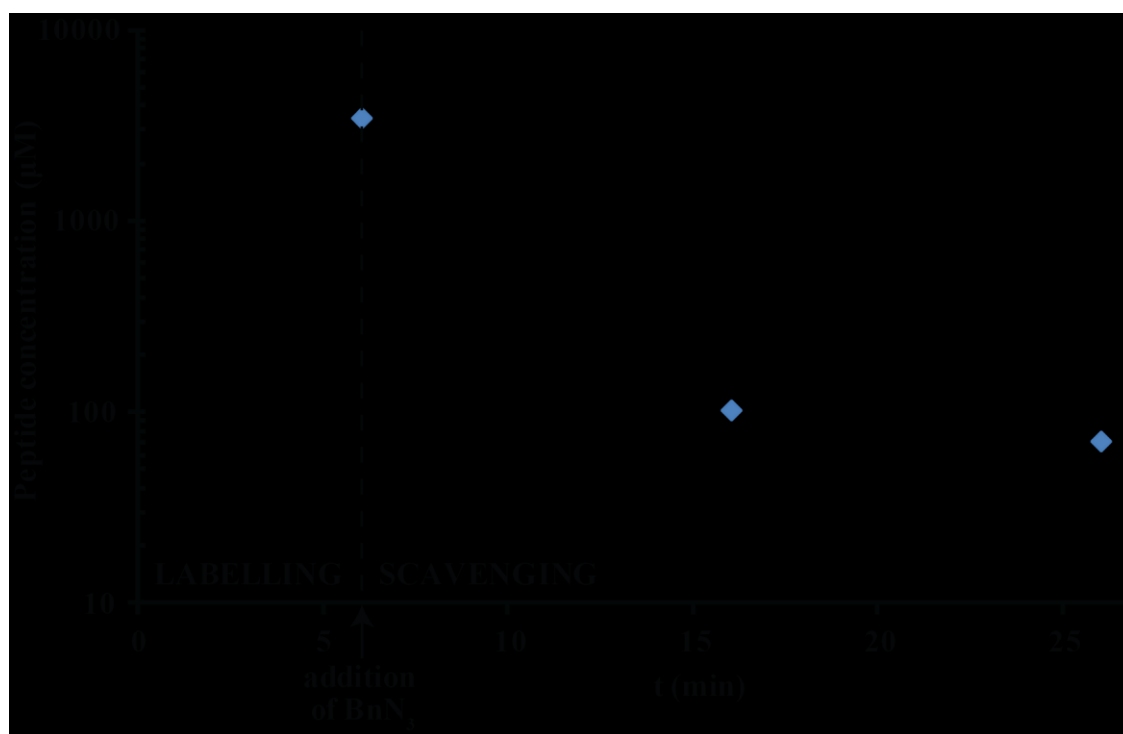


Figure 27. Evolution over time of the peptide concentration during the scavenging process. BnN_3 was added to the reaction mixture at $t = 6$ min. Peptide concentration was calculated from the product's area under the curve on the reaction mixture's UV-trace. Data are mean of two independent experiments.

The experiments showed that the apparent peptide concentration, obtained from the integration of the UV-HPLC traces and a calibration curve, decreased from 3.48 mM to 103 μM in 10 min and to 71.2 μM in 20 min ($n = 2$). In other words, this represents a potential improvement of the apparent specific activity by a factor of 34 in 10 min and by a factor of 49 in 20 min ($n = 2$). It is important to note that, to the best of our knowledge, the improvement of specific activity by chemical means has never previously been achieved. However, we believe that the method described above is of wide applicability as the lipophilicity of the scavenger can be adjusted easily in order to fit the particular requirements of the tracer in preparation. For instance, the difference in retention time between the tracer and the scavenged precursor could be increased by the use of a more lipophilic azide, such as azidomethylnaphthalene. The concept of scavenging is also applicable to other prosthetic groups: it is therefore theoretically possible to scavenge unreacted hydroxylamine-containing precursors (that could be labelled with *para*- ^{18}F fluorobenzaldehyde) with lipophilic aldehydes. Finally, and as aforementioned, the concept is not limited to HPLC separation, but can be applied to fluorous-SPE for instance, as demonstrated below.

2.3.3. Perfluorinated scavenging

Similar scavenging procedure with $n\text{-C}_8\text{F}_{17}\text{CH}_2\text{CH}_2\text{N}_3$, readily synthesised by displacement of the corresponding commercial iodide with NaN_3 , occurred at a slightly lower reaction rate, affording a potential improvement of the apparent specific activity by a factor of 23 in 10 min and by a factor of 31 in 20 min. After loading the thus scavenged reaction mixture onto a fluorous-SPE cartridge, the release of the radioactivity was performed with various mixtures of MeOH–water containing 25 mM HCl (Figure 28).

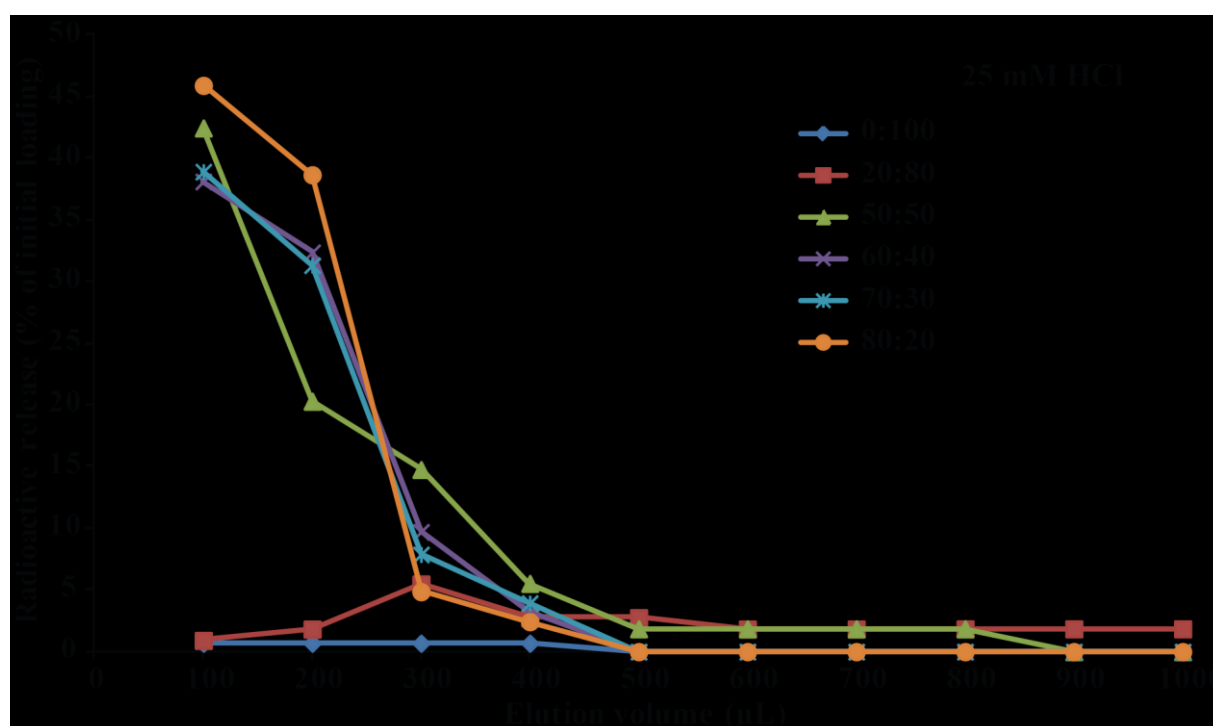


Figure 28. Elution of the radioactivity from the fluorous-SPE cartridge.

The cartridge was eluted in $10 \times 100 \mu\text{L}$ fractions for each experiment and the activity of each fraction was recorded as the percentage of the activity initially bound to the fluorous-SPE. A content of 50% MeOH in acidic water was found minimal to allow release of the tracer from the fluorous-SPE cartridge, thus allowing maximal release in less than $500 \mu\text{L}$. Analysis of the decayed $100 \mu\text{L}$ fractions by mass spectrometry showed that no breakthrough of scavenged peptide could be witnessed at 50% and 60% MeOH, whereas presence of perfluorinated peptide was detected from fraction 3 with 70% and from fraction 2 with 80%

MeOH. Thus, the 60% MeOH eluate consisted of the tracer, the hydrophilic species (such as copper and sodium ascorbate) and the accelerating ligand, with no trace of scavenged peptide.

Going further in our investigation, we wanted to be able to remove the hydrophilic species and the accelerating ligand with an SPE cartridge, as this would provide us an HPLC-free purification of the tracer, allowing the design of a completely automated synthesis of the tracer from fluoride directly. Dilution of the 60% MeOH eluate with water and loading this solution onto a preconditioned Waters tC₁₈ cartridge allowed trapping of the radioactivity. A non-radioactive attempt at evaluating the required elution strength to remove compound **30** from a tC₁₈ cartridge showed that 300 μ L 20% MeOH in acidic water was sufficient to do so. Therefore, the cartridge was washed with 1 mL of this mixture, releasing only negligible amounts of radioactivity. Finally, 52% of the radioactivity could be eluted with 200 μ L of 25 mM HCl in EtOH (Figure 29).

Although the scavenging step and the purification by fluorous-SPE worked extremely well, some ligand **30** could still be observed after tC₁₈ SPE. Nevertheless, although the ligand's peak is the major peak in the UV chromatogram of the purified sample, its strong UV absorbance belies an actually low amount of accelerating ligand; Figure 29C shows for comparison the intensity of the ligand's UV peak before purification. Therefore, although the tC₁₈ SPE purification step did not allow for complete purification of the reaction mixture, we could not afford to improve this further. This could be done by washing the tC₁₈ SPE cartridge with a larger amount of 20% MeOH in aqueous acid or 30% MeOH in aqueous water should achieve complete removal of compound **30**, thus affording the desired radiotracer in good purity without the use of any HPLC purification.

However, our desire to assess the biological characteristics of our tracer made us focus on the benzyl azide scavenging technique at this juncture.

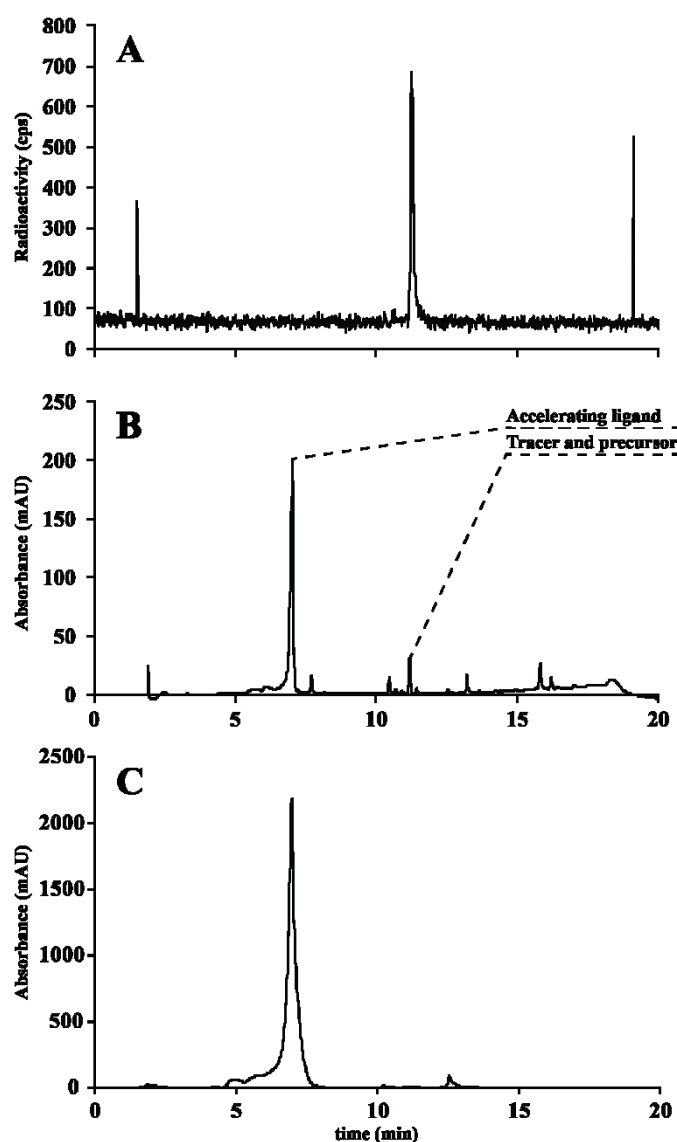


Figure 29. Representative chromatograms for the scavenging method with the perfluorinated azide after purification by fluororous-SPE and tC₁₈ SPE, sequentially (no semi-preparative HPLC). A: radiotracer of the purified fraction. B: UV-trace of the purified fraction. C: UV-trace of the crude reaction mixture after benzyl azide scavenging, for comparison of the size of the peak corresponding to the accelerating ligand.

2.4. Radiosynthesis and further biological evaluation of the tracer

2.4.1. Radiosynthesis

Using the optimised scavenging method with BnN₃, the labelled peptide [¹⁸F]**28** was produced in approximately 30 min with an apparent specific activity of 19.4 GBq.μmol⁻¹ and the decay-corrected yield was 63±16% (*n* = 5) starting from 178 MBq [¹⁸F]FEA. Overall, the EOS-RCY was of 18±6% (*n* = 5) in approximately 1.5 h from aqueous [¹⁸F]fluoride. Analytical HPLC

showed a radiochemical purity >98% and that the tracer was stable in 10% (v/v) EtOH–PBS for at least 4 h at r.t. (Figure 30).

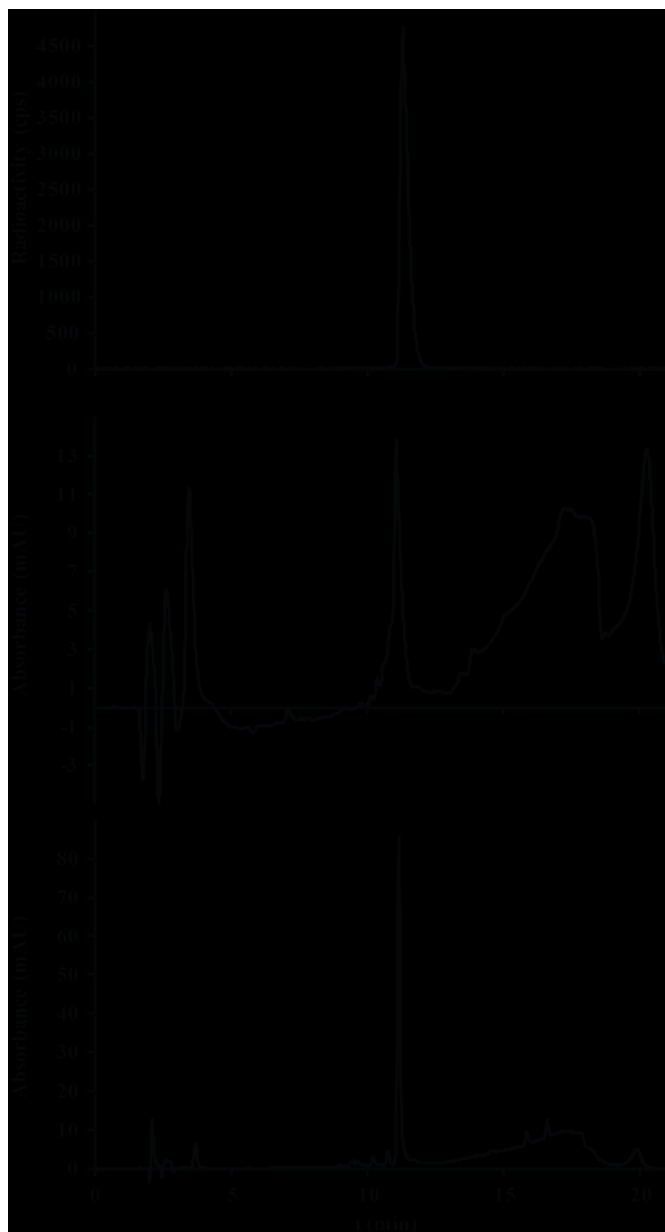


Figure 30. Quality control of the purified delivered $[^{18}\text{F}]\mathbf{28}$. A: radioactive trace of the formulated tracer. B: UV chromatogram of the formulated tracer. C: UV chromatogram of the tracer spiked with $[^{19}\text{F}]\mathbf{28}$.

The distribution coefficient of the tracer $[^{18}\text{F}]\mathbf{28}$ was also evaluated. The distribution ratio at physiological pH of blood serum was assessed by measuring the distribution of radioactivity between *n*-octanol and PBS (pH 7.4) using the “shake flask” method.¹⁴⁹ The logarithm of the

ratio was calculated as $\log D_{\text{pH } 7.4} = -0.11 \pm 0.02$ ($n = 5$). This balanced lipophilicity was assumed to be appropriate for a short-lived radiotracer as it should account for an optimal renal elimination,¹⁵⁰ faster pharmacokinetics and therefore increased contrast.¹⁵¹

Dr. Federica Pisaneschi performed deliveries of this tracer for biological evaluation.

2.4.2. Cellular uptake assay

Cellular uptake of [^{18}F]**28** was assessed in U87.CD4 cells transfected with CXCR4 (U87.CD4.CXCR4) (Figure 31).

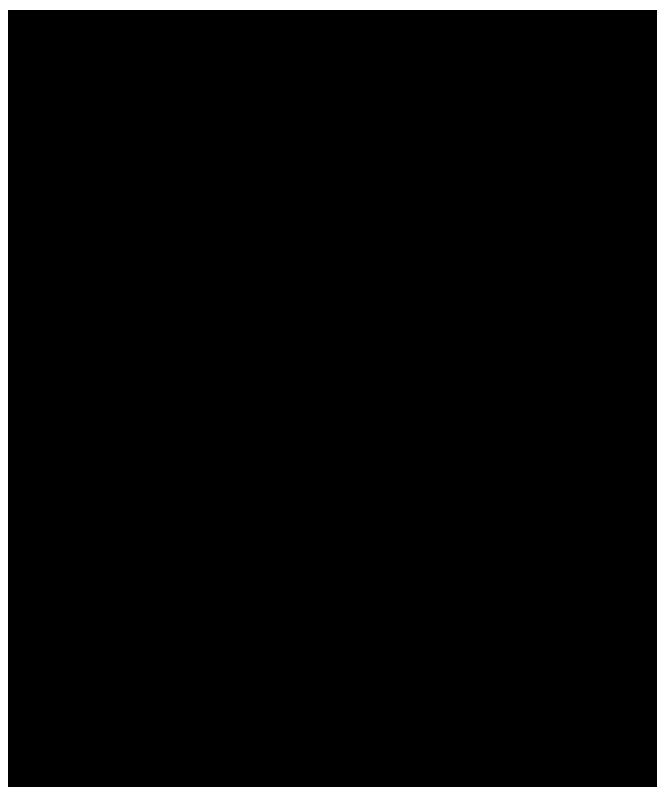


Figure 31. [^{18}F]**28** *in vitro* uptake in U87.CD4 and U87.CD4.CXCR4 cells. Data are mean $\pm$ SD of three independent experiments and statistical significance was confirmed using a 2-way ANOVA with Bonferroni post-tests ($P < 0.05$).

60-min cellular uptake of [^{18}F]**28** showed a two-fold higher uptake in the CXCR4-expressing cells compared to isogenic CXCR4-low cells, confirming potency of the tracer to CXCR4 as seen in the competitive binding assay. Furthermore, addition of 20 μM of FC131 showed a decrease of the cellular uptake to the level of the CXCR4-low cells control, demonstrating

specificity of the tracer for CXCR4. These biological data, along with the IC_{50} value measured for our tracer, validated our SAR analysis, and we therefore decided to assess the tracer *in vivo*.

2.4.3. Biodistribution

U87.CD4.CXCR4 tumour cells were xenografted to BALB/c nu/nu athymic nude mice and when the tumour reached at least 80 mm³ the mice were injected with the tracer. At 60 min post-injection, the mice were sacrificed, tissues were harvested and their radioactivity counted (Figure 32).

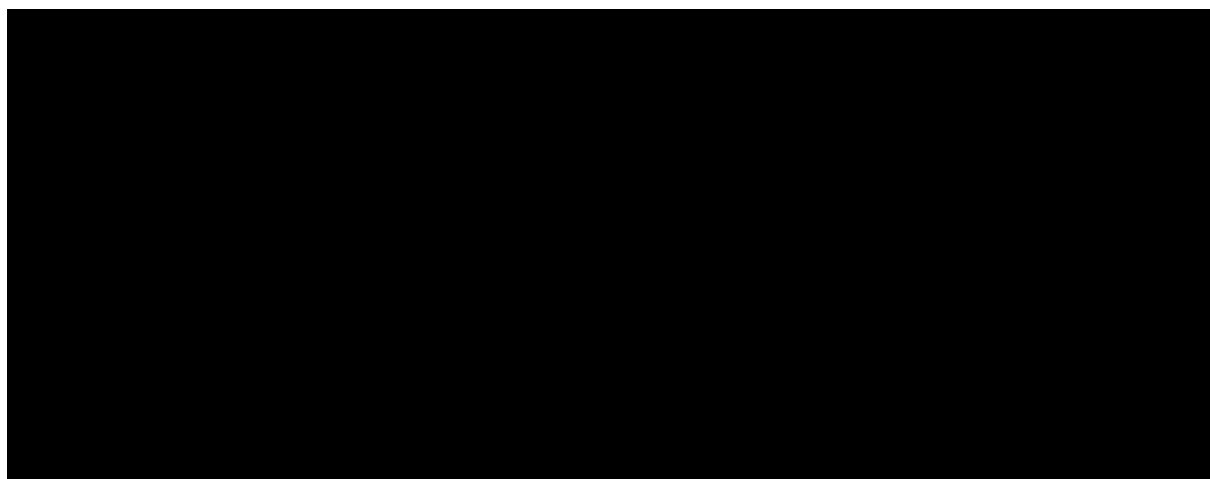


Figure 32. Tissue distribution of [¹⁸F]**28** in U87.CD4.CXCR4-tumour bearing mice at 60 min post-injection. Data are mean $\pm$ SEM of three independent experiments.

The main radioactivity uptake was found in the urine, sign of renal elimination. Some activity was also found in the liver and gallbladder indicating limited excretion *via* the hepatobiliary route and metabolic activity. Unfortunately, the tumour showed only little uptake, presenting a tumour-to-muscle ratio of approximately 1, meaning that it would not be possible to distinguish between the tumour and envioning tissues. Moreover, some uptake was found in the bone suggesting possible *in vivo* defluorination, although the low uptake in the tumour might just expose it more markedly by comparison.

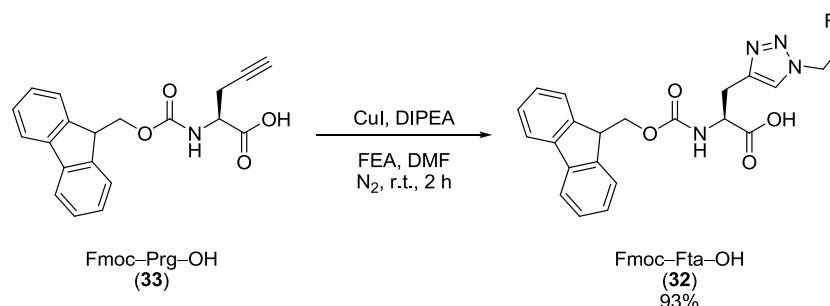
2.5. Conclusion

Analogue **28** showed good affinity in *in vitro* assays (radioligand competitive binding assay with the non-radioactive isotopologue and cellular uptake assay with [^{18}F]**28**) with an IC_{50} value of $1.04\ \mu\text{M}$ (compared to FC131: $0.65\ \mu\text{M}$) and a 2-fold uptake in U87.CD4.CXCR4 tumour cells. This advocated for compound **28** to be a potent and CXCR4-specific tracer. However, *in vivo* validation of the tracer showed poor biodistribution data with only negligible uptake in the tumour compared to the uptake in the muscle. With three more candidate tracers devised from our SAR analysis and the docking study, we chose to assess their biological properties in the anticipation that, provided that they show similar or superior *in vitro* characteristics, they might present an improved *in vivo* biodistribution pattern.

3. Other candidate tracers

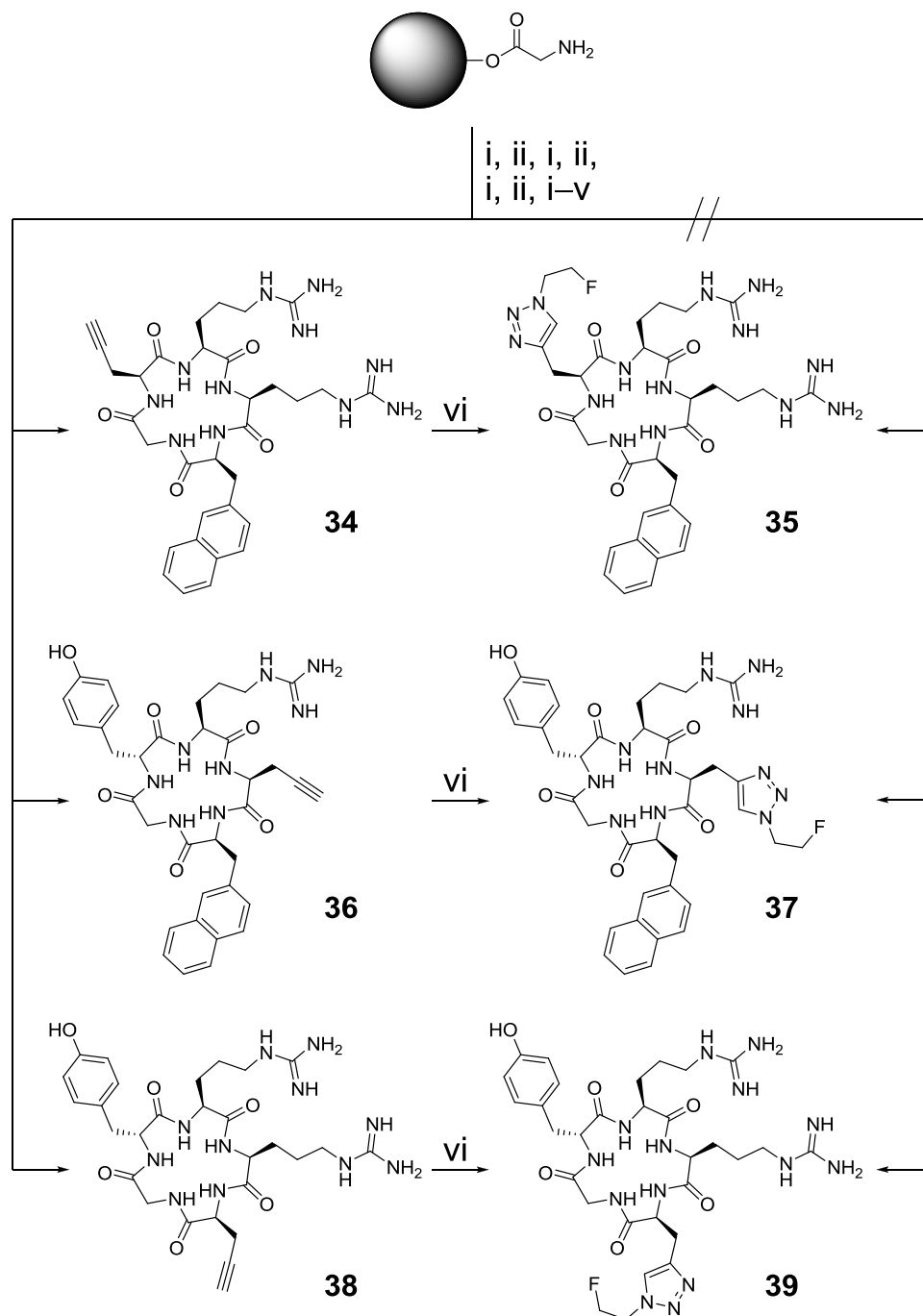
3.1. Synthesis of an array of cyclopentapeptides: precursors and reference compounds

The synthesis of the six additional precursors and reference compounds was undertaken by systematically substituting FC131's residues, with the exception of Gly1, for either Fta [1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-ylalanine] or Prg. In an attempt to accelerate the synthesis, we wanted to be able to synthesise the six corresponding peptides in parallel. This should be achievable by parallel solid-phase peptide synthesis with non-proteogenic Fmoc-Fta-OH (**32**). Fmoc-Fta-OH was synthesized from commercially available Fmoc-protected propargyl glycine (**33**) *via* copper(I)-mediated alkyne-azide cyclisation with FEA in excellent yields (Scheme 16).



Scheme 16. Synthesis of Fmoc-Fta-OH (**32**).

Solid-phase peptide synthesis was carried out with both of these non-proteogenic amino acids to introduce the desired acetylenic and fluoroethyltriazolyl side-chains (Scheme 17).



Scheme 17. Synthesis of FC131 analogues, precursors and reference compounds. Conditions: (i) Fmoc-Xaa-OH, HOBt, HBTU, DIPEA, DMF, r.t., 2 h; (ii) 20% piperidine in DMF, r.t., 30 min; (iii) AcOH-TFE-DCM (1:1:3), r.t., 2 h; (iv) NaHCO₃, DPPA, DMF, -40 °C to r.t., 4 days; (v) TFA-TIS-H₂O (190:5:5), r.t., 6 h; (vi) CuSO₄·5H₂O, sodium ascorbate, FEA, sodium acetate buffer (pH 5, 250 mM), DMF, r.t., N₂, 7 days.

Alkyne-containing analogues were readily obtained in overall yields of 12–45%. In regard of the number of steps required to synthesise these precursors, this corresponds to the good to excellent yield of 82–93% per single transformation.

Although the deprotection conditions used are common for peptide synthesis (TFA–TIS–H₂O, 190:5:5), their application to the linear protected triazole-containing pentapeptides surprisingly proved to be too harsh, resulting in complex mixtures of unidentified by-products and the isolation of insufficient quantities of the desired reference compounds. Instead, reaction of their alkyne counterparts **34**, **36** and **38** by Huisgen alkyne-azide cycloaddition with FEA followed by preparative HPLC purification efficiently afforded the desired products in excellent (89%) to quantitative yields.

The affinity of the thus prepared reference compounds was assessed by radioligand competitive binding assay (Table 11).

Table 11. Results of the binding affinity and migration assays for FC131 and our four analogues.

Entry	Compound	Structure	IC ₅₀ (μM)	
			Binding	Migration
1	FC131	<i>cyclo</i> (–Gly–D–Tyr–Arg–Arg–Nal–)	0.65±0.22	0.042±0.012
2	35	<i>cyclo</i> (–Gly– <u>F</u> ta–Arg–Arg–Nal–)	0.37±0.12	>1000
3	28	<i>cyclo</i> (–Gly–D–Tyr– <u>F</u> ta–Arg–Nal–)	1.04±0.28	11.4±3.90
4	37	<i>cyclo</i> (–Gly–D–Tyr–Arg– <u>F</u> ta–Nal–)	19.9±7.06	>1000
5	39	<i>cyclo</i> (–Gly–D–Tyr–Arg–Arg– <u>F</u> ta–)	>100	–

Data are **mean** ± SEM of three independent experiments.

To our delight, analogue **35** proved to be more potent than the original FC131. This can be explained by the size of the triazole ring, smaller than that of D-Tyr, which can therefore fit in the same pocket and thus preserve all of the interactions the other residues and the cyclopentapeptidic backbone make with CXCR4. It also cannot be excluded that one of the triazole's nitrogen participates in the binding *via* hydrogen bond with CXCR4's Tyr190 and the aromatic ring itself *via* hydrophobic contacts, mimicking D-Tyr2's interactions with the receptor (Figure 19C). On the other hand, compound **37**, although less potent than **28**, showed

some unexpectedly good affinity. This again could be accounted for by the possibility of the triazole moiety to hydrogen bond with Asp187 and the fluorine atom with Asp97. Finally, NaI5's substitution drastically decreased the affinity of our ligand for CXCR4, confirming our hypothesis that it is the second aromatic ring of the naphthalene moiety that is in contact with His203 *via* π -stacking.

It is worth noting that these experimental results are in good accordance with the SAR analysis and the docking studies previously described. Analogue **35**, exhibiting superior affinity for our target receptor, is a good candidate for radiolabelling and further *in vitro* and *in vivo* study. It can be anticipated that such an improvement in the IC₅₀ value would reduce the reversibility of its binding to CXCR4, making it less available for renal excretion or hepatic metabolism.

3.2. Radiosynthesis and further biological evaluation of the tracer

3.2.1. Radiosynthesis.

The radiosynthesis of [¹⁸F]**35** was performed by Dr Federica Pisaneschi. Using the optimised scavenging method with BnN₃, the labelled peptide [¹⁸F]**35** was produced in approximately 50 min with an apparent specific activity of 6.7 GBq.μmol⁻¹ and the decay-corrected yield was 55±11% (*n* = 3) starting from 178 MBq [¹⁸F]FEA (Figure 33).

Overall, the non-decay-corrected EOS-RCYs was of 19±6% (*n* = 3) in approximately 1.5 h from aqueous [¹⁸F]fluoride. Analytical HPLC showed a radiochemical purity >98% and that the tracer was stable in 10% EtOH–PBS for at least 4 h at room temperature.

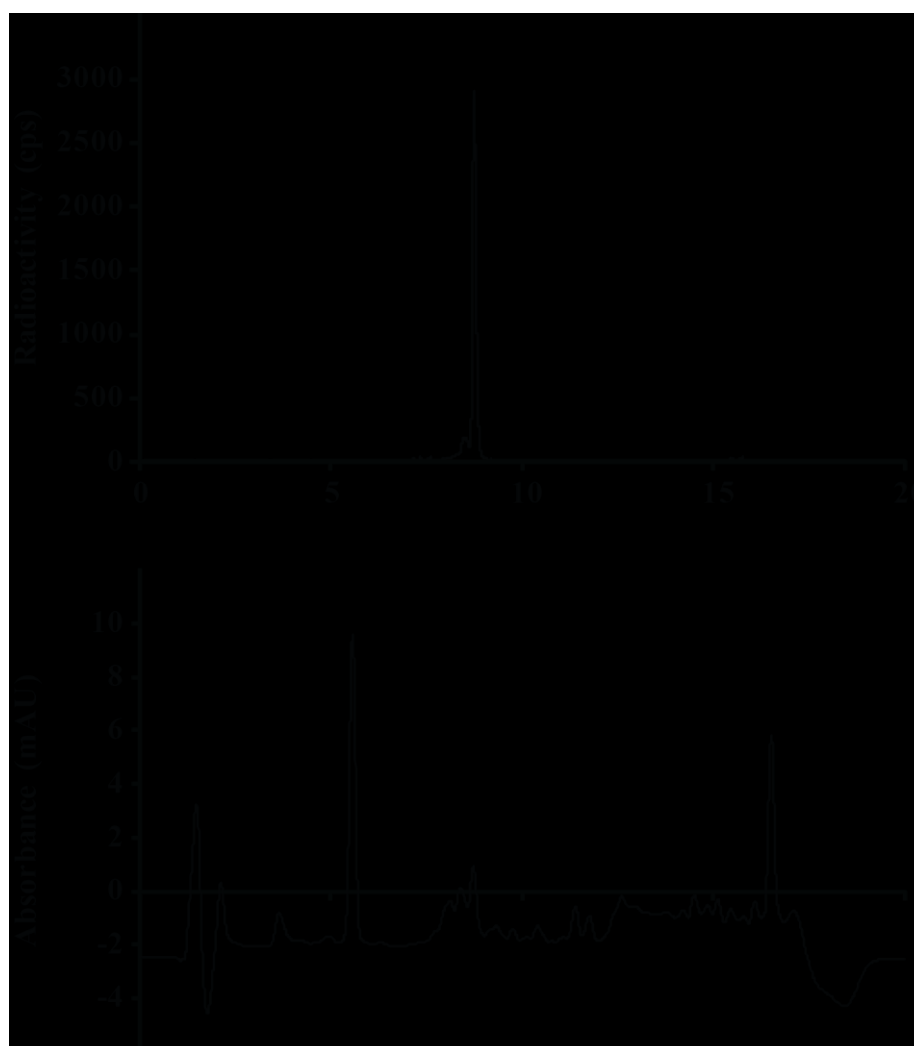


Figure 33. Quality control of the delivered [^{18}F]**35**. A: radioactive trace of the formulated tracer. B: UV chromatogram of the formulated tracer.

3.2.2. Cellular uptake assay

Cellular uptake of [^{18}F]**35** was assessed in U87.CD4 cells transfected with CXCR4 (U87.CD4.CXCR4) (Figure 34).

60-min cellular uptake of [^{18}F]**35** showed a two-fold higher uptake in the CXCR4-expressing cells compared to isogenic CXCR4-low cells. Furthermore, addition of 20 μM of FC131 showed a decrease of the cellular uptake to the level of the CXCR4-low cells control, demonstrating specificity of the tracer for CXCR4. This radiotracer therefore showed very comparable cellular uptake characteristics to that of [^{18}F]**28**.

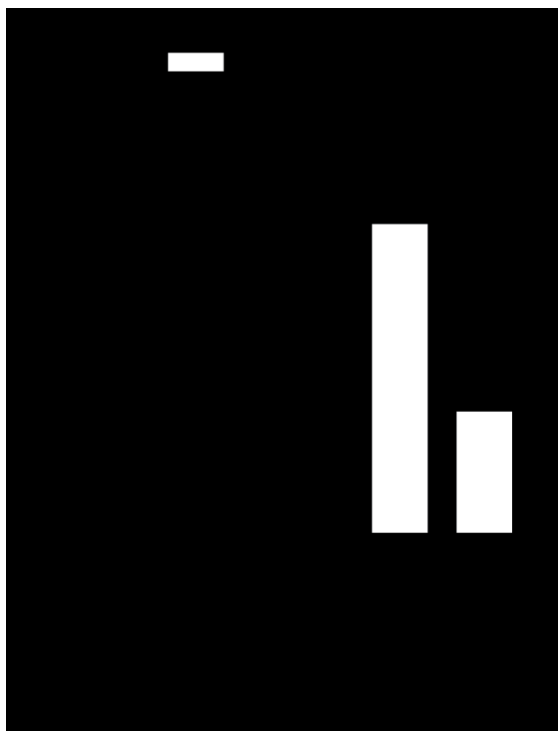


Figure 34. [¹⁸F]35 *in vitro* uptake in U87.CD4 and U87.CD4.CXCR4 cells. Data are mean ± SD of three independent experiments and statistical significance was confirmed using a 2-way ANOVA with Bonferroni post-tests ($P < 0.05$).

3.2.3. Imaging and biodistribution

U87.CD4 and U87.CD4.CXCR4 tumour cells were xenografted to BALB/c nu/nu athymic nude mice and when the tumour reached at least 80 mm³ the mice were injected with the tracer and scanned over 60 min (Figure 35 and Figure 36).

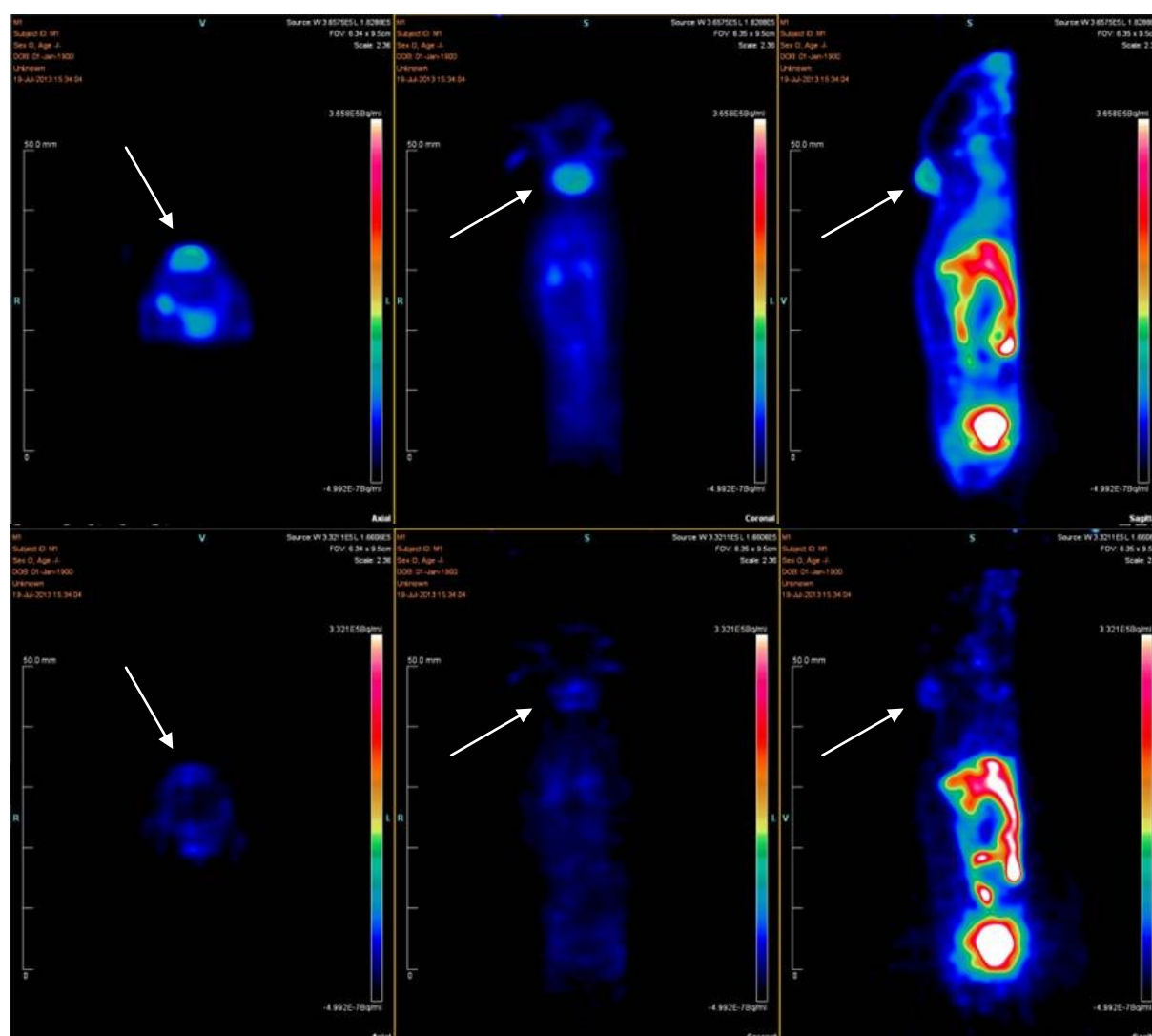


Figure 35. Dynamic CT/PET imaging of 3.7 MBq of [^{18}F]**35** in mice bearing U87.CD4.CXCR4-derived tumours. Top: Averaged over 5–30 min post-injection. Bottom: Frame at 55 min post-injection. Left: Axial section. Middle: Frontal section. Right: Sagittal section. White arrowheads indicate the tumours.

From the imaging data averaged over 5–30 min (Figure 35, top), the tumour presents some contrast with the background. However, a large amount of activity is detected in the bladder and in the intestine. Moreover, the imaging frame at 55 min shows no uptake of radioactivity in the tumour anymore, which means that the tracer is washed out of the tumour before the end of the scan. This behaviour was further confirmed by the time *versus* activity curve, which additionally exhibits no difference in uptake between the positive and the negative cell lines (Figure 36).

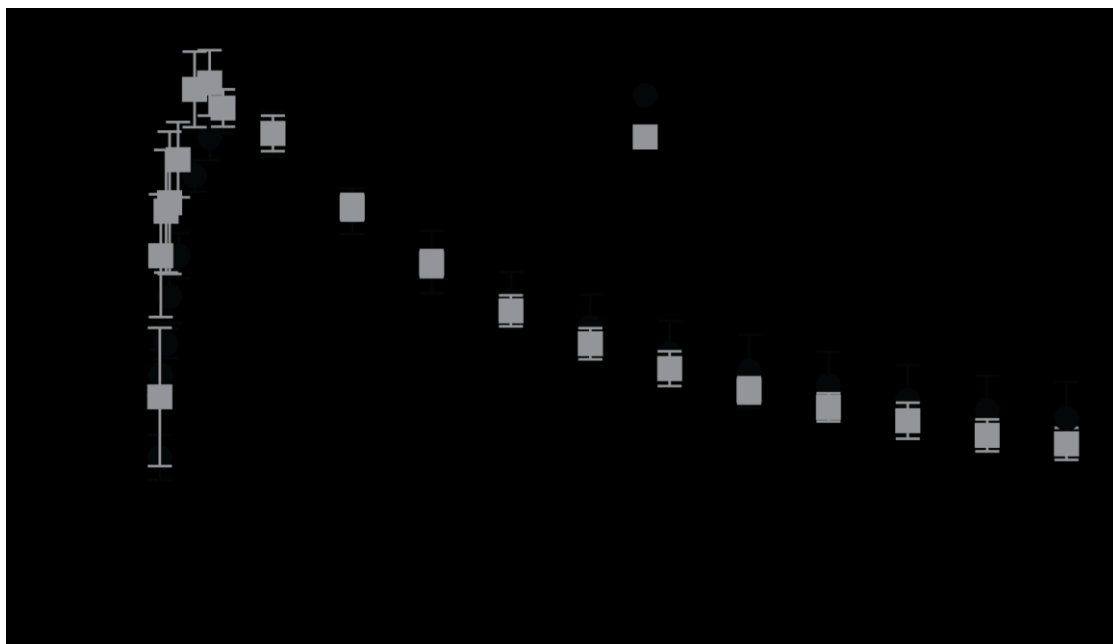


Figure 36. Tumour time *versus* activity curves for the dynamic CT/PET imaging of 3.7 MBq of [^{18}F]35 in mice bearing either U87.CD4- or U87.CD4.CXCR4-derived tumours. Data are mean $\pm$ SEM.

After reaching a maximum in ~ 4 min, the activity found in the tumours slowly decreased exponentially with a half-life of ~ 20 min. Both cell lines showed very similar behaviour. Although this could theoretically be explained by non-specific binding, it is highly unlikely as the tracer uptake of U87.CD4.CXCR4 tumour cells could be blocked *in vitro* by the pre-incubation with FC131. Another, and maybe more plausible explanation, is that the denser vasculature found in tumours is synonymous of a larger concentration of blood in the tumour compared to the rest of the body, thus explaining the apparent tracer uptake in the both the positive and the negative cell lines.

At 60 min post-injection, the mice were sacrificed, various tissues were harvested and their radioactivity counted (Figure 37).

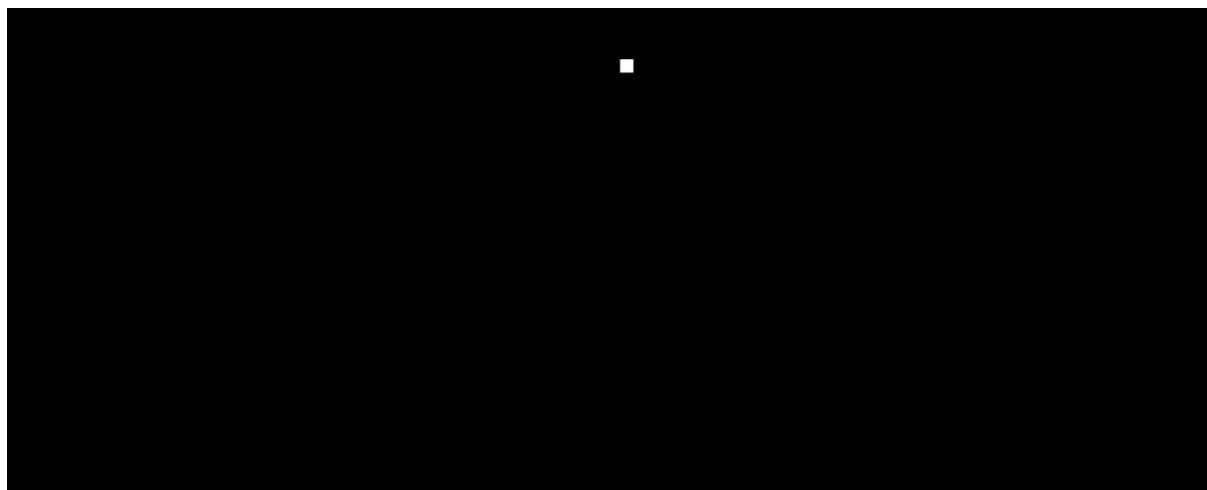


Figure 37. Tissue distribution of [^{18}F]**35** in U87.CD4- or U87.CD4.CXCR4-tumour bearing mice at 60 min post-injection. Data are mean $\pm$ SEM.

The biodistribution data thus collected was similar to that found for [^{18}F]**28**. The main uptake was found in the urine and then in the gallbladder and the liver, which can be justified by renal excretion, hepatobiliary excretion and hepatic metabolism, respectively. Again, no difference between the two cell lines was witnessed.

With a tracer presenting promising *in vitro* properties, it was necessary to understand the biological mechanisms that lead to the lack of uptake observed *in vivo* in order to elude them. These data can be rationalised in two ways. Our first hypothesis was that the tracer is rapidly metabolised by the liver and that metabolites are quickly excreted *via* the kidneys. Our second hypothesis was that the tracer was directly excreted *via* the urinary route, without prior metabolism.

These two hypotheses could be tested by studying the presence of metabolites in the plasma, the liver and the urine.

3.2.4. Metabolite study

The radioactive content of plasma, urine and liver at 10, 30 and 60 min post-injection were partially monitored as previously reported (see section II.4.4) (Table 12 and Table 13).

Table 12. Metabolite study of [¹⁸F]**35**. Representative radiochromatograms for plasma, urine and liver preparations at 10, 30 and 60 min post-injection.

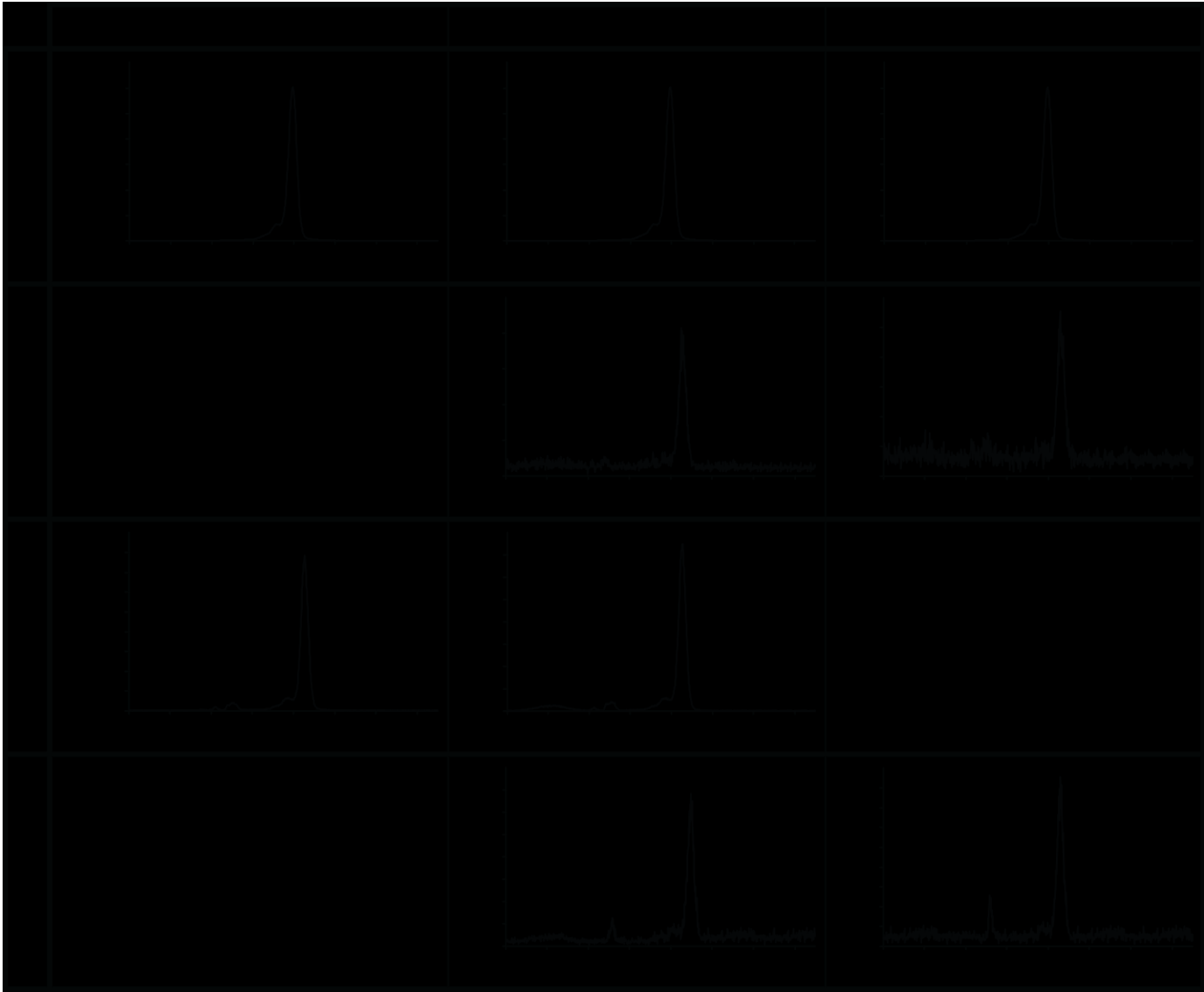


Table 13. Metabolite study of [¹⁸F]**35**. Proportion of parent compounds in plasma, urine and liver samples at 10, 30 and 60 min post-injection.

	Plasma	Urine	Liver
10 min	n.d.	94%	n.d.
30 min	95%	85%	91%
60 min	92%	n.d.	88%

Proportion defined as $\frac{AUC_{\text{parent compound}}}{AUC_{\text{parent compound}} + AUC_{\text{metabolites}}}$. n.d.: not determined.

Although the data presented are partial and based on a single experiment, it can be concluded that tracer [¹⁸F]**35** is stable *in vivo* for lengths of time relevant to PET. Thus, 92% of the radioactivity found in plasma at 60 min post-injection corresponded to the parent compound. Similarly, the tracer seemed stable toward liver metabolism, with only 12% of the radioactivity present in this organ coming from metabolites. Expectedly, the highest levels of radioactivity were found in the urine, where the tracer was found in abundance (85% at 30 min post-injection). It is therefore clear the low tumour uptake measured is due to excessive renal excretion of the fluorine-18-labelled tracer. Although we could not afford to perform a metabolite study for [¹⁸F]**28**, we assume that the discrepancy between *in vitro* and *in vivo* tumour uptake was probably for analogous reasons.

4. Conclusions

Based on the careful study of the FC131 analogues found in the literature, we have been able to build an SAR analysis of the CXCR4 pocket where cyclopentapeptides bind. Our analysis was confirmed by docking studies using the appropriate crystal structure and docking variables, thus allowing us to build strong assumptions of the binding mode of FC131. From those two independent analyses, it appeared that the main interactions made by FC131, or *cyclo*(–Gly1–D-Tyr2–Arg3–Arg4–Nal5–), are performed by the basic arginines *via* salt bridges and hydrogen bonds with aspartate and glutamate residues. In particular, Arg3 was the residue the most likely to accept modifications thanks to its good solvent exposure and because it points towards the extracellular milieu. Secondary interactions, in the form of hydrophobic contacts, are performed by naphthylalanine and D-tyrosine. Although this last residue is likely to face steric hindrance, its replacement of a small aromatic residue was

conceivable. Finally, Gly1 was deemed important for the overall conformation of the cyclopentapeptidic backbone and thus for the orientation and resulting interactions of its residues with the receptor.

In order to confirm our analysis, we undertook the synthesis of four candidate tracers and their corresponding precursors by systematically substituting FC131's residues – except for Gly1 – by Fta, a triazole-containing non-proteogenic amino acid of our devise, and Prg, a commercially available acetylenic amino acid, respectively. Assessment of the affinity of the thus produced reference compounds, by radioligand competitive binding assay and migration assay, showed that two of them, **28** and **35**, showed equivalent to improved binding potency for CXCR4 compared to FC131.

The radiosynthesis of these two analogues was carried out with [^{18}F]FEA and the corresponding alkyne-bearing precursors with a copper(II) – sodium ascorbate – accelerating ligand system in excellent conversion within 10 min. Nevertheless, this resulted in poor specific activity of the delivered material, owing to the impossibility to separate the prepared tracer for the precursor used in excess. This issue is often met for the labelling of large molecules such as peptides because the physico-chemical properties of the resulting tracers and precursors are so comparable that they do not allow their separation by preparative HPLC. We undertook the design of a method to improve the specific activity, which took the form of a one-pot and rapid chemical scavenging of the unreacted acetylenic functional group. Carried out with benzyl azide, which is readily available in one step from commercial benzyl bromide in excellent yield, we managed to reduce the concentration of precursor in the reaction mixture by a factor of 34 in 10 min and 49 in 20 min by simply adding BnN_3 in the reaction mixture after the labelling was complete. Consequently, [^{18}F]**28** was afforded in $19.4 \text{ GBq} \cdot \mu\text{mol}^{-1}$ in ~30 min starting from 178 MBq [^{18}F]FEA. Similarly, we were able to use a perfluorinated azide to carry out the scavenging step and to remove the resulting scavenged peptide by mere fluorous-SPE.

Evaluation of the *in vitro* characteristics of our two tracers proved positive, with a 2-fold uptake in the CXCR4-positive cell lines compared to CXCR4-low cell lines. Moreover, this uptake was blockable by pre-incubation of the cells with FC131, demonstrating that both tracers bind potently and specifically to CXCR4.

Unfortunately, these promising results were not confirmed *in vivo*. Although uptake in the CXCR4-expressing U87.CD4.CXCR4 tumours could be detected, detection of the same level

of uptake in the CXCR4-low U87.CD4 cell lines suggested it was not related to the expression of CXCR4 but due to the increased vascularity of tumours. Moreover, large amounts of radioactivity in the bladder indicated potential rapid excretion *via* the renal route. Tracer [^{18}F]**35** was found to be stable *in vivo*, with only limited amount of metabolite found in the plasma and liver 60 min post-injection. Analysis of the origin of the radioactivity found in urine confirmed our hypothesis that excessive rate of tracer renal excretion preclude tumour uptake.

The favourable *in vitro* data and overall *in vivo* stability validate our approach in the design and synthesis of a CXCR4-specific fluorine-18-labelled probe based on the structure of cyclopentapeptidic FC131. The high urinary excretion rate observed accounts for the lack of *in vivo* tumour uptake; preclusion of such excretion can therefore be expected to allow for tumour uptake.

CHAPTER IV.

SMALL MOLECULES

1. Motivations and selection of suitable derivatisations

1.1. Literature analogues

As described by Thoma *et al.*,⁵⁰ the first compound of the isothioureia class to be found active against CXCR4 was IT1a (Figure 38).

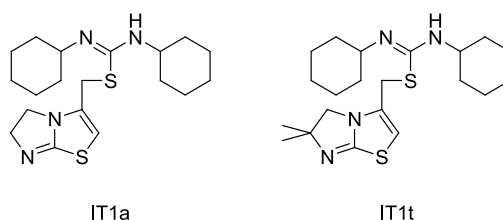


Figure 38. Structures of IT1a and IT1t.

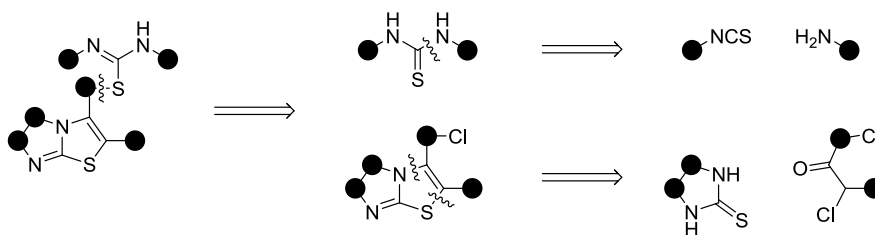
IT1a was discovered by screening the Novartis compound library in a radioligand binding assay. Further investigation of this lead compound showed high selectivity for CXCR4 over other GPCRs and chemokine receptors, very good pharmacokinetic properties, such as a good water solubility, low intrinsic clearance when treated with human microsomes and a slow elimination rate in the rat model.

It is worth noting that this compound is protonated at physiological pH at both the isothioureia nitrogens and at the dihydroimidazothiazole nitrogen; these positive charges are likely to create beneficial electrostatic interactions with the negatively charged binding site of the chemokine receptor. This was evidenced by methylation of one of the isothioureia's nitrogens,

which substantially decreased the potency, showing that it is also required for hydrogen bonding.⁵⁰

In the same article, the screening of different analogues is reported. Compounds obtained by substitution of the cyclohexyl rings for larger cycloalkanes (cycloheptyl, cyclooctyl) displayed more potent CXCR4 antagonism while the 2-adamantyl analogue retained some satisfactory activity. Conversely, substitution of a cyclohexyl ring for a 1-adamantyl moiety reduced the activity. These observations suggest that CXCR4 can accommodate a rather large lipophilic group *in lieu* of one of the cyclohexyl groups. Furthermore, addition of two methyl groups on the dihydroimidazothiazole core, thus affording analogue IT1t, improved both the ligand's binding affinity and its *in cellulo* Ca^{2+} mobilisation antagonism while retaining favourable pharmacokinetic properties.

Finally, the synthesis described in the paper⁵⁰ makes this class of compounds very attractive as all the analogues were readily available upon refluxing the corresponding thioureas with the appropriate chloride followed by recrystallation in good yield and purity. From this, chemical routes to a variety of precursors allowing the introduction of radioisotopes at different positions can be devised by simply modifying one or several of four building blocks, allowing the rapid construction of a library of PET tracer candidates (Scheme 18).



Scheme 18. Retrosynthetic analysis of the IT1t class of compounds. Available positions for the introduction of chemical diversity are depicted as black balls.

The $\text{CH}_2\text{--S}$ bond of IT1t analogues can be created by reacting a thiourea with the dihydroimidazothiazole core bearing a leaving group such as a benzylic chloride. In turn, the thiourea can be produced by reaction of an amine with an isothiocyanate. In parallel, the dihydroimidazothiazole core can be synthesised by treating another thiourea with a derivative of 1,3-dichloroacetone.

Although undoubtedly IT1t is a superb starting point for the design of new small molecule PET tracers specific for CXCR4 and of remarkable pharmacokinetic profile, no firm conclusions about the most promising disconnection to explore could be drawn from the work presented by Thoma *et al.*⁵⁰ Therefore, further investigation of the local environment of this class of compounds when bound to CXCR4 was desirable.

1.2. Derivatisation site selection

As mentioned previously, IT1t was recently co-crystallised with CXCR4.¹² Analysis could bring valuable insights in the binding mode and the scope of possible modifications that could be envisioned to be introduced into this type of molecule (Figure 39).

As described by Stevens *et al.*,¹² the strongest interactions between IT1t and CXCR4 are the two salt bridges formed between the two of the three protonated nitrogens of IT1t and Asp97, Glu288 and the hydrogen bond between the other protonated nitrogen of IT1t and one of the backbone's carbonyl in CXCR4. This further supports the hypothesis that both thiourea nitrogens are key to the binding of the ligand to CXCR4. Secondary lipophilic interactions are detected for the dihydroimidazothiazole core (Trp94 and Tyr116), one of the cyclohexyl (hydrophobic interactions with Ile185 and Arg183) and the other cyclohexyl (sitting in a hydrophobic pocket made of Cys186, Trp94, Trp102 and Val112).

However considerable the knowledge a crystal structure can provide, in the case of a GPCR the structural plasticity of the receptor makes analysis even more challenging. To date, the majority of GPCRs have been crystallised as oligomers. This is because GPCRs are known to form such oligomeric structures on cell surfaces potentially resulting in different downstream signalling pathways to those from monomeric GPCRs.^{13,14} Although still an area of active research, it is apparent that the physical structures of the same GPCR (and thus of its binding site) as a monomer and as an oligomer differ significantly.

Cognisant of this situation, we decided to carry out a general approach to portray the main and recurrent interactions between IT1t and CXCR4. For this purpose, we chose to dock *in silico* IT1t in its binding site in order to compare the different poses the ligand is able to adopt.

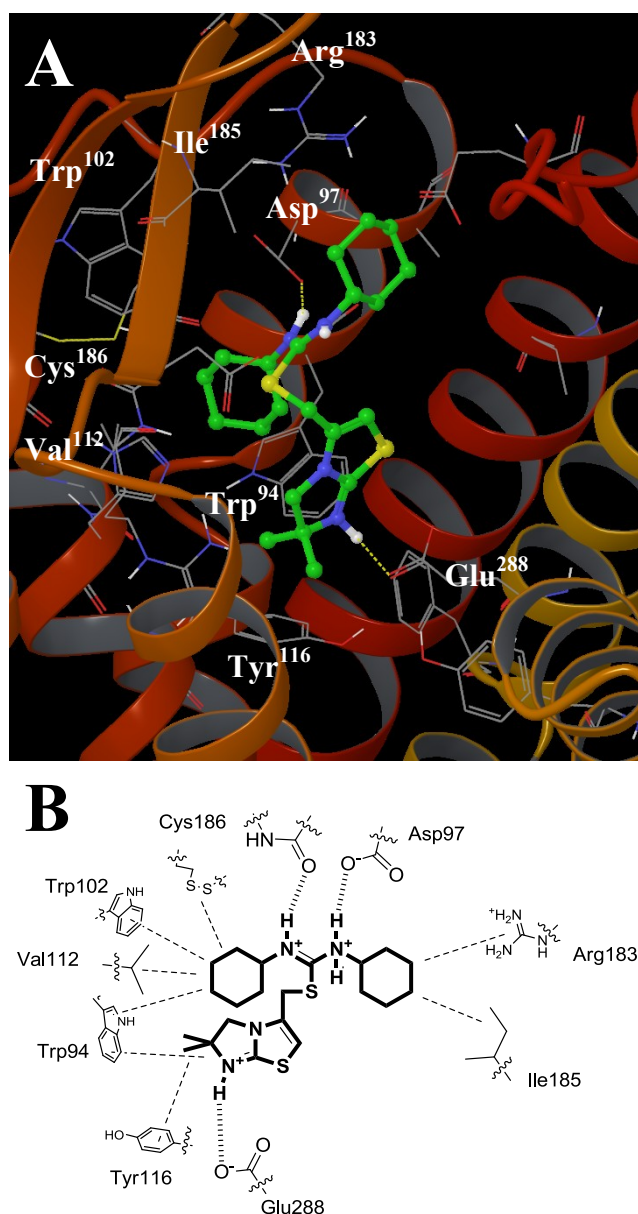


Figure 39. Interactions between CXCR4 and IT1t as evidenced in the co-crystal structure. A: As can be seen using Schrödinger software, hydrogen bonds are depicted as yellow dotted lines. B: Schematic representation, salt bridges and hydrogen bonds are represented as thick dash bonds, hydrophobic interactions are represented as thin dash bonds.

The *in silico* work described in this chapter was carried out on the Schrödinger software package¹⁴⁰⁻¹⁴⁴ and the appropriate crystal structure for CXCR4 (PDB: 3OE6).¹² The receptor was prepared with the Protein Preparation Wizard tool to correct structural problems inherent to crystal structures (*e.g.*, residue overlaps, protonation states, disulfide bridges, bond-order assignments) and a docking grid containing the entire receptor's binding site was created with default settings. IT1t was extracted from the crystal structure and treated with the Epik tool in

order to find the most accurate protonation state under biological conditions. Energy minimisation was performed with the LigandPrep tool, thus yielding 248 conformations of lower energy. The 24 conformations (10%) of lowest energy of these conformations were docked *in silico* with the Glide tool into the docking grid previously defined, resulting in 192 poses.

The docking results showed that all of the poses are within the same binding pocket as the co-crystallised ligand, thus validating the docking preparation work and confirming the particular affinity between IT1t and this particular pocket. Furthermore, 59 poses presented the same primary interactions with CXCR4 to the co-crystallised ligand pose, namely salt bridges between Glu288 and the dihydroimidazothiazole's positively charged nitrogen and between Asp97 and the isothioureia's positively charged nitrogen.

It was then possible to compare these poses by stacking them onto a single image (Figure 40).

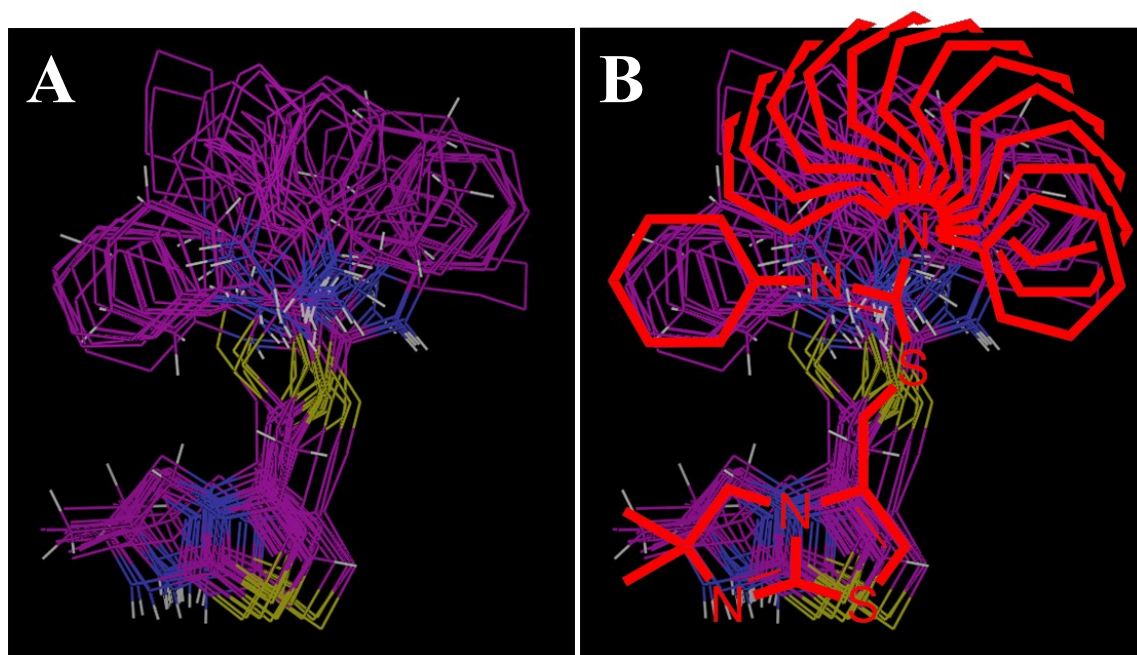


Figure 40. Overlay of 39 poses of IT1t in CXCR4. A: As seen using Schrödinger. B: With a schematic representation of IT1t overlaid.

This computational study provided useful insight. First, it can be clearly seen that the dihydroimidazothiazole core is rather well defined within the pocket, as well as one of the cyclohexyls. This confirms what could be inferred from the crystal structure itself, namely

that the molecular interactions that are important for the binding of IT1t to CXCR4 are two-fold. On the one hand, the salt bridges between Asp97 and Glu288 and the ligand's positively charged nitrogens and on the other hand the non-polar interactions between Tyr116 and the dihydroimidazothiazole core and between CXCR4's hydrophobic pocket formed by Trp94, Val112, Trp102 and Cys186 and one of the ligand's cyclohexyl groups.

Second, it can be observed that the second cyclohexyl ring does not seem to have any preferential position within the binding site. This finding confirms what was noticed from the crystal structure (Figure 39): the interactions of this cyclohexyl ring with CXCR4's residues (Arg183 and Ile 185) are fewer than that of the other cyclohexyl ring and probably less crucial for the binding affinity. Therefore, this particular moiety was selected for modification in order to allow the introduction of a radioisotope.

More recently, a few analogues of IT1t were patented (Figure 41).¹⁵²

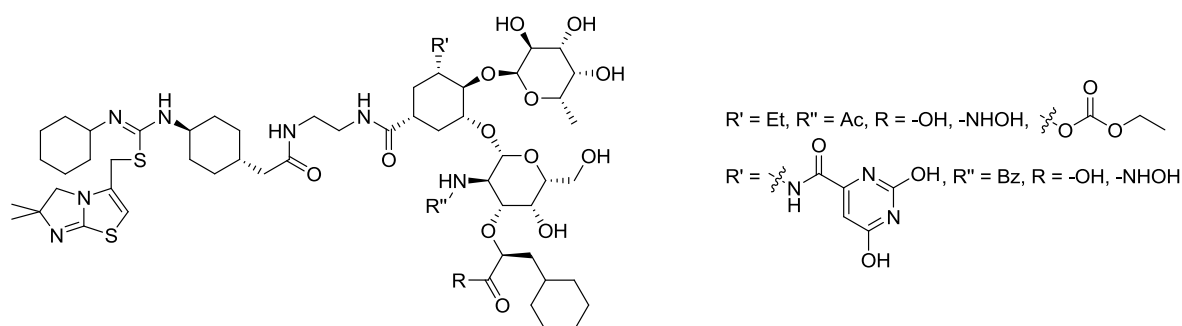


Figure 41. Example of IT1t analogues patented by Magnani *et al.*¹⁵²

Although none of these structures are associated to any biological data, it can be thought that the inventors' choice to modify one of the cyclohexyl groups for glycomimetic moieties supports our own conclusions about the most promising region of the molecule for modification.

1.3. Options for derivatisation

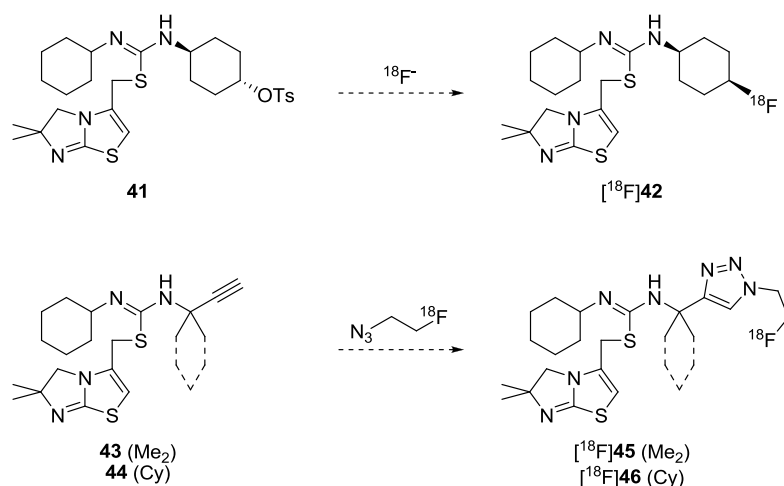
As aforementioned (see section I.5), there are many ways to introduce a radioelement into an organic molecule.

The introduction of fluorine-18 could be carried out directly either by displacement of a leaving group such as a tosylate or a mesylate, or by S_NAr on a precursor bearing an adequately electron-withdrawing group on the aromatic ring. This approach has the advantage of being single-step, but it is generally difficult to employ on more sophisticated molecules. The alternative approach is the use of prosthetic groups. Although this strategy entails a multistep, *i.e.* a longer, radiosynthetic route, the synthesis of the prosthetic group *per se* is usually mastered and its reaction with the precursor generally requires little optimisation. [^{18}F]FEA (for click chemistry with alkynes),⁷⁶ 5- ^{18}F fluoropent-1-yne (^{18}F **40**) (for click chemistry with azides)⁷⁵ and *p*- ^{18}F fluorobenzaldehyde (**14**) (for oxime formation with *O*-hydroxylamines¹⁵³ or for reductive amination with amines¹⁵⁴) are some of the most widely used prosthetic groups for introduction of fluorine-18.

In contrast, radiometals have the advantage that their chelation into a precursor is generally fast and clean. However, their use requires the introduction of a chelating group into the precursor's structure. These chelating groups are generally rather large, especially when compared to small molecules and can substantially change the physicochemical and biological properties of the compound. This is why radiometals are normally reserved for the labelling of larger molecules such as peptides, proteins, antibodies or peptide nucleic acids.⁹⁰

We decided to first assess the affinity of simple analogues of IT1t for CXCR4 before exploring the synthesis of more synthetically challenging tracers (Scheme 19).

The initial investigation began with the synthesis of a fluorocyclohexyl analogue (**42**), which exhibits minimal structural modification to IT1t. This was envisaged to constitute a proof of principle for fluorine-18-labelled IT1t analogues used as CXCR4-specific PET tracers. In parallel the synthesis of two alkyne-bearing precursors (**43** and **44**) for click-labelling with [^{18}F]FEA was examined. We deployed this strategy previously on cyclopentapeptides and it proved efficient (see chapter III).



Scheme 19. Precursors for radiofluorination by $\text{S}_{\text{N}}2$ (**41**) and by click chemistry with [^{18}F]FEA (**43** and **44**) and resulting tracers ([^{18}F]42, [^{18}F]45 and [^{18}F]46).

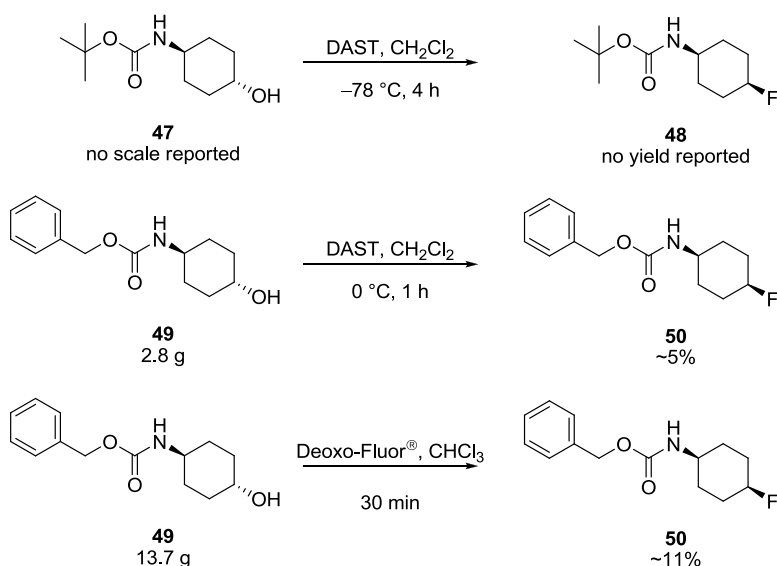
2. Cyclohexyl and propargyl analogues

2.1. Cyclohexyl analogues

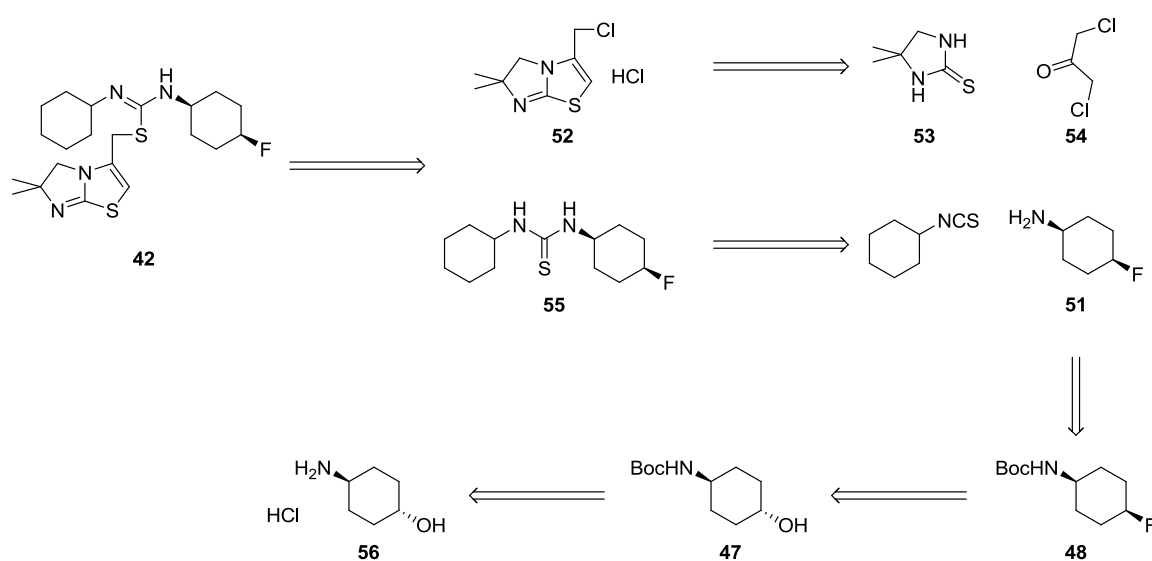
2.1.1. Synthesis of the reference compound

The most challenging step in the formation of the cold fluorocyclohexyl derivative **42** was anticipated to be the introduction of fluorine. As a matter of fact, very few instances of the synthesis of 4-fluorocyclohexylamine are found in the literature; all three of them in patents (Scheme 20).¹⁵⁵⁻¹⁵⁷

In all cases, the fluorine atom was introduced by reaction of a cyclohexanol derivative with a deoxyfluorination reagent such as diethylaminosulfur trifluoride (DAST)^{155,156} or *bis*(2-methoxyethyl)aminosulfur trifluoride (Deoxo-Fluor®).¹⁵⁷ Although these two reagents are known to efficiently perform deoxyfluorination on a wide variety of substrates, it is worth noting the poor yields associated with this transformation in the case of *N*-protected 4-hydroxycyclohexylamines **47** and **49** to afford **48** and **50**, respectively. Nevertheless, owing to the low amount of reference compound required for preliminary biological evaluation (binding affinity and migration assay) and radiochemistry (spiking of the HPLC trace to confirm the identity of the tracer), we decided to include this step in our synthetic route (Scheme 21).



Scheme 20. All literature instances of fluorination towards the synthesis of 4-fluorocyclohexylamine (51).¹⁵⁵⁻¹⁵⁷

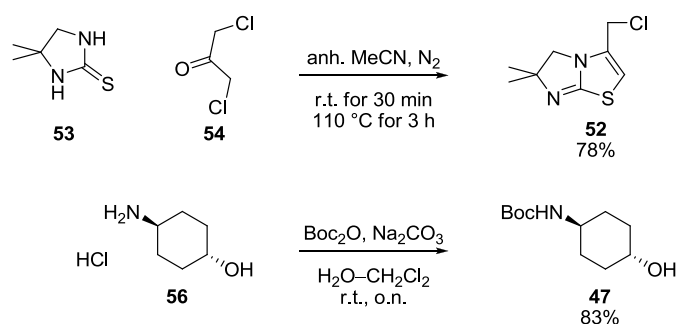


Scheme 21. Retrosynthetic analysis towards the fluorocyclohexyl IT1t analogue **42**.

As aforementioned (Scheme 18), isothiurea **42** could be accessed by reacting thiourea **55** with chloride **52**, which could in turn be obtained by treating thiourea **53** with 1,3-dichloroacetone **54**. In parallel, formation of thiourea **55** could be achieved by the reaction of CyNCS with amine **51**, which can be produced by the deprotection of *tert*-butyl-carbamate **48**. Finally, this carbamate can be synthesised by Boc-protection of *trans*-4-

hydroxycyclohexan ammonium chloride **56** followed by deoxyfluorination as shown in Scheme 21.

Chloride **52** was obtained following a minor modification to the literature procedure of Thoma and co-workers⁵⁰ as a pure white precipitate in good yields (78%) (Scheme 22).

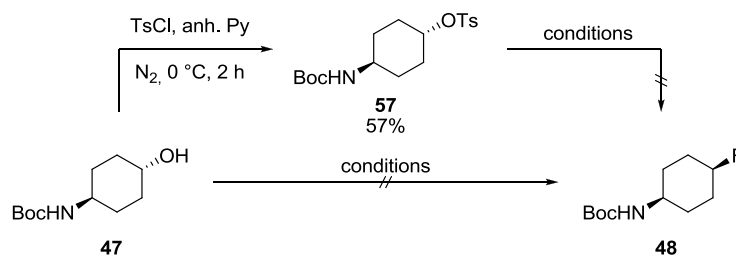


Scheme 22. Synthesis of chloride **52** and alcohol **47**.

In addition, treatment of ammonium chloride **56** with Boc_2O and Na_2CO_3 in a biphasic $\text{H}_2\text{O}-\text{CH}_2\text{Cl}_2$ system afforded alcohol **47** in 83% yield after recrystallisation from EtOAc.

A deoxyfluorination reaction comprises two fundamental steps. First, the electrophilic centre of the deoxyfluorinating agent is attacked by the alcohol to form a good leaving group. Second, nucleophilic fluoride attacks this leaving group to afford the corresponding fluorinated compound.

Various attempts at carrying out the deoxyfluorination step on **47** were performed (Scheme 23, Table 14).

Scheme 23. Attempts at deoxyfluorinating **47**. Conditions are detailed in Table 14.Table 14. Attempts at deoxyfluorinating **47**.

Entry	Starting material	Procedure	Outcome
1	Alcohol 47	DAST, 0 °C	Elimination
2	Alcohol 47	DAST, -40 °C	Elimination
3	Alcohol 47	DAST, -78 °C	Elimination
4	Alcohol 47	Deoxo-Fluor [®] , -78 °C	Elimination
5	Alcohol 47	XtalFluor-E [®] , -78 °C	Elimination
6	Alcohol 47	XtalFluor-M [®] , -78 °C	Elimination
7	Alcohol 47	XtalFluor-E [®] , DBU, -78 °C	Elimination
8	Alcohol 47	XtalFluor-M [®] , DBU, -78 °C	Elimination
9	Tosylate 57	CsF, anh. MeCN, r.t.	Elimination
10	Tosylate 57	CsF, anh. tBuOH, 90 °C	Elimination
11	Tosylate 57	KF, anh. ethylene glycol, 85 °C	Elimination

Following a first procedure from the literature,¹⁵⁶ treatment of alcohol **47** with DAST at 0 °C afforded only the elimination product (entry 1). This could be easily determined by inspection of ¹⁹F and ¹H NMR spectra. No signal was detected in the ¹⁹F NMR spectrum. Analysis of the ¹H NMR spectrum showed two signals around $\delta = 5.60$ ppm, each integrating for 1.0 proton, with a roof effect, correlating with what could be expected for an ABXY system (Figure 42).

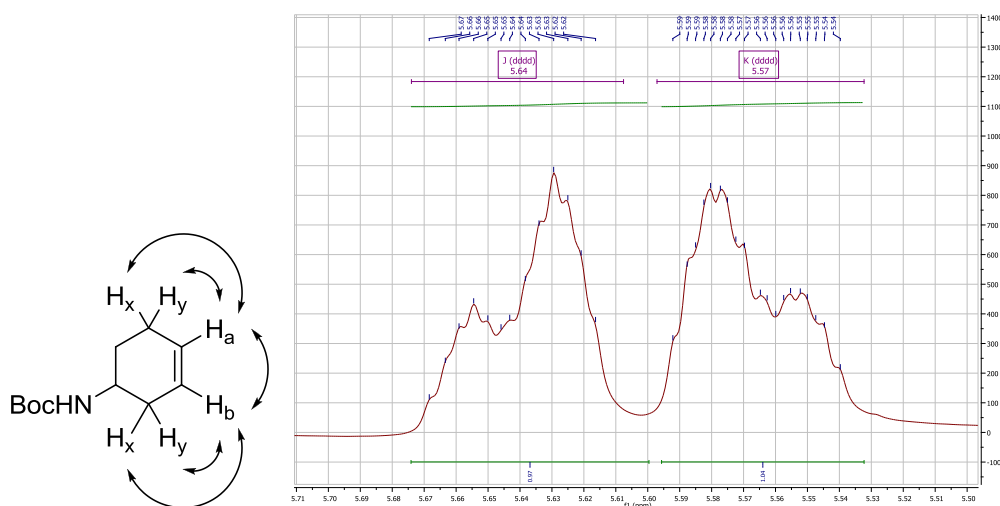


Figure 42. Representation of the two ABXY systems present in the elimination product and ^1H NMR spectrum at $\delta = 5.70\text{--}5.50$ ppm.

Efforts to reduce the reaction temperature to $-40\text{ }^\circ\text{C}$ or $-78\text{ }^\circ\text{C}$ in order to prevent the elimination occurring made no difference in the outcome (entries 2–3), and neither did the substitution of DAST for Deoxo-Fluor[®] (entry 4). The use of XtalFluor-E[®] [(diethylamino)difluorosulfonium tetrafluoroborate] and XtalFluor-M[®] [difluoro(morpholino)sulfonium tetrafluoroborate], the milder and more selective alternatives to DAST and Deoxo-Fluor[®],¹⁵⁸ was also ineffective. Their use with or without 1,8-diazabicycloundec-7-ene (DBU), a non-nucleophilic base that has been shown to improve deoxyfluorination yields when used as an additive in this type of reaction by deprotonating the *N,N*-dialkylaminodifluorosulfane intermediate and preventing release of diethylamine, was ineffective as well (entries 5–8).

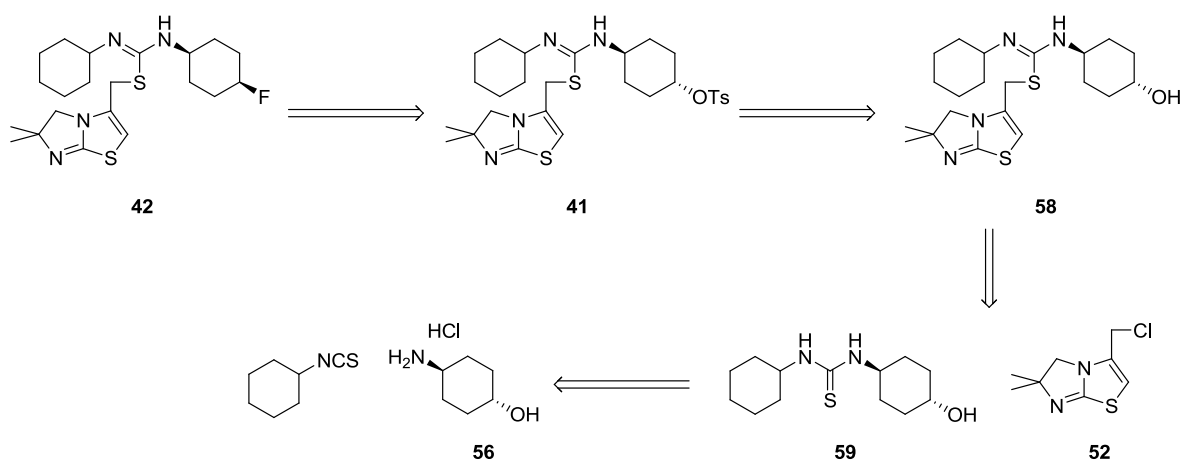
Following unsuccessful attempts at optimising deoxofluorination, it was decided to carry out the fluorination by nucleophilic substitution of the tosylate derivative of alcohol **47** with fluoride anion. To this end, tosylation of alcohol **47** afforded the desired product in moderate yields (Scheme 23). Treating tosylate **57** with CsF at room temperature in anhydrous MeCN yielded only the elimination product once again (entry 9). Because elimination and substitution are in competition in this reaction, we sought to explore substitution conditions in order to favour the formation of the desired compound **48**. To this end, an attempt at carrying out the reaction in refluxed *tert*-butanol was performed (entry 10).⁶¹ This solvent is deemed to form "flexible" fluorides of higher nucleophilicity than CsF and to temper its basicity, thus theoretically improving the yield of our reaction. Furthermore, *tert*-butanol may also

hydrogen bond the tosylate moiety, thus improving its leaving group ability. Finally, this protic solvent can inhibit other possible side-reactions (such as elimination or substitution with other nucleophiles, including eventual remaining traces of water) by hydrogen bonding these other nucleophiles. Unfortunately, only elimination product could be detected in the inspection of the ^1H NMR spectrum of the crude reaction mixture. Attempts to use ethylene glycol as a solvent for similar reasons¹⁵⁹ also did not yield any product (entry 11).

2.1.2. Synthesis of the precursor

The synthetic challenge outlined above prompted a change in our approach. We sought to synthesise tosylate **41** as a precursor. This was done in the expectation that although elimination would still likely happen during the fluorination step, the very particular conditions under which radiochemistry is performed would likely render this pathway still acceptable. Even considering that only a small fraction of the precursor does not undergo elimination and is available for nucleophilic substitution, that small fraction should still represent a large excess compared to the amount of ^{18}F fluoride used for radiolabelling. Thus, excellent RCYs could still be anticipated.

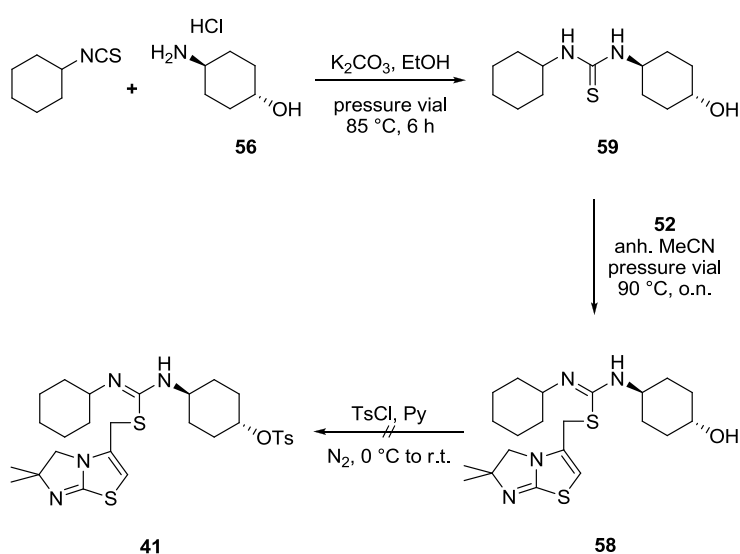
The presence of the strongly nucleophilic thiourea's sulfur and of the good leaving group ability of tosylates prompted modification to the synthetic strategy. The introduction of the tosylate functional group would follow the reaction between the corresponding thiourea and chloride **52** (Scheme 24).



Scheme 24. Retrosynthetic analysis towards the fluorocyclohexyl IT1t analogue **42** via precursor **41**.

Compound **42** could be accessed by displacement with fluoride of the tosylate group in precursor **41**, which in turn could be synthesised from alcohol **58** and TsCl. The alcohol could be obtained from the reaction of thiourea **59** with chloride **52** made previously. The tosylate could in turn be synthesised from the alcohol and finally, CyNCS could react with ammonium chloride **56** to afford the required thiourea **59**.

Thiourea **59** was obtained in quantitative yield by reaction of CyNCS, ammonium chloride **56** and K₂CO₃ in EtOH (Scheme 25).



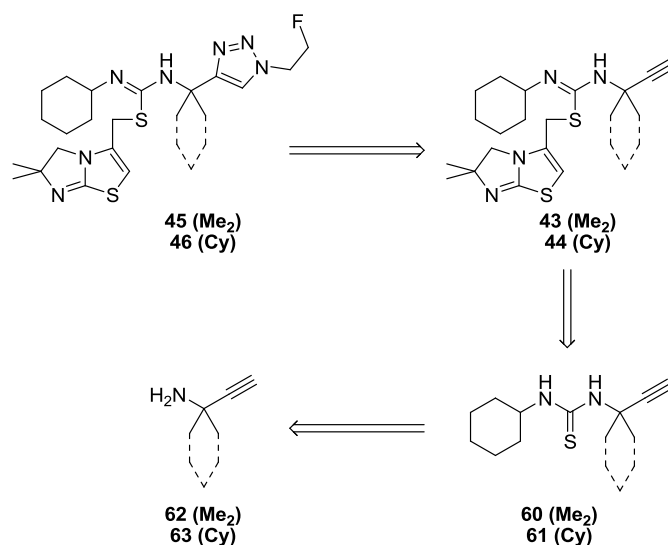
Scheme 25. Synthesis of alcohol **58** and its attempted tosylation.

Thiourea **59** readily reacted with chloride **52** to afford alcohol **58** in excellent purity as a white precipitate. However, the formation of tosylate **41** proved more challenging than expected. Treatment of alcohol **58** with TsCl in pyridine did not afford the desired product. Monitoring the reaction by HPLC and mass spectrometry (MS) indicated that the starting material was being consumed; however, no product could be detected by mass spectrometry. The ¹H NMR spectrum of the crude reaction mixture was difficult to interpret due to the presence of many impurities. Attempts to purify the crude product by flash column chromatography, preparative TLC, semi-preparative HPLC and recrystallisation were unsuccessful. In view of the manifold synthetic obstacles encountered this far, at this juncture we resolved to focus on the alkyne-containing analogues instead.

2.2. Propargyl analogues

2.2.1. Synthesis of the precursors

As indicated earlier (Scheme 19), one of the main aims was to synthesise two precursors based on the structure of IT1t for radiolabelling by click chemistry with [^{18}F]FEA (Scheme 26).

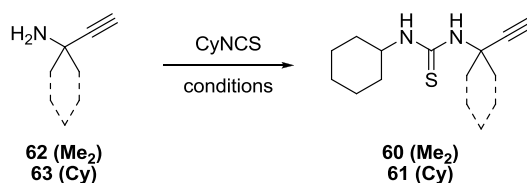


Scheme 26. Retrosynthetic analysis towards two alkyne-containing IT1t analogues.

In order to retain a structure relatively analogous to that of IT1t, we originally designed compound **44** with two cyclohexyl rings. However, the combination of a cyclohexyl and a triazole ring could potentially interfere with the binding of the tracer. This consideration led to the synthesis of dimethyl analogue **43**, which presents a less sterically demanding footprint.

Reference compounds **45** and **46** could be obtained by [3+2]cycloaddition between FEA and alkynes **43** and **44**, respectively. In turn, these alkynes can be synthesised by reacting chloride **52** with thioureas **60** and **61**, respectively. Finally, the treatment of the corresponding amines **62** and **63** with CyNCS should afford the required thioureas.

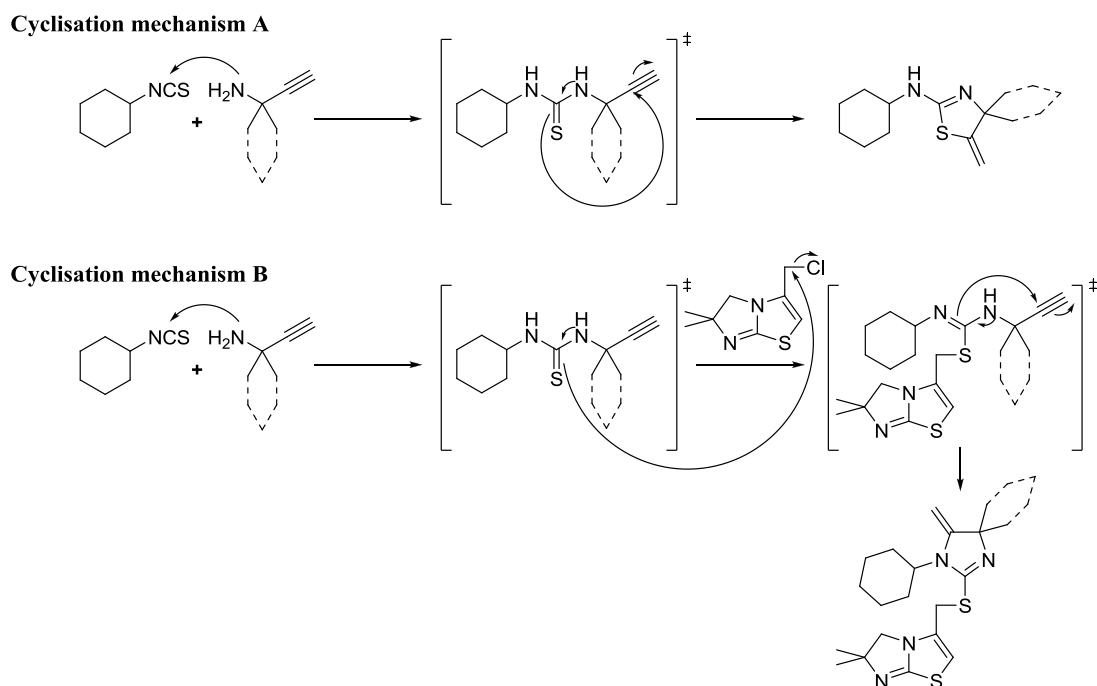
Numerous attempts at carrying the first step were performed as outlined in Table 15.

Table 15. Attempts at forming thioureas **60** and **61**.

Entry	Starting material	Solvent	Additive	Purification method	Outcome ^f
1	63	anh. MeCN	–	SiO ₂ ^b	cyclisation A
2	62	anh. MeCN	–	SiO ₂ ^b	cyclisation A
3	63	anh. MeCN	anh. NEt ₃	SiO ₂ ^b	cyclisation A
4	63	EtOH	–	SiO ₂ ^b	cyclisation A
5	63	EtOH	–	Al ₂ O ₃ ^c	cyclisation A
6	63	EtOH	anh. NEt ₃	SiO ₂ + NEt ₃ ^d	cyclisation A
7	63	EtOH	one-pot ^a	HPLC ^e	cyclisation B
8	62	EtOH	one-pot ^a	HPLC ^e	cyclisation B

All attempts were carried out at 1 M, r.t, N₂, o.n.. ^a after o.n. reaction, 1 equiv. **52** was added and the reaction mixture was stirred for 1 week at r.t.. ^b flash column chromatography on silica. ^c flash column chromatography on basic alumina. ^d flash column chromatography on silica with 0.1% NEt₃. ^e preparative HPLC, gradient 15–30% B over 20 min. ^f see Scheme 27.

The two amines **62** and **63** in anhydrous MeCN were treated with CyNCS (entries 1–2); however, the only product that could be isolated by flash column chromatography was an unexpected product in ~40% yield. Particularly useful for the resolution of the structure of this compound was analysis of the ¹H NMR spectrum, which presented a signal of an AB system at $\delta = 5.10$ and 5.06 ppm ($J = 1.6$ Hz) with roof effect, coupling to a triplet ¹³C at $\delta = 101.3$ ppm. This corresponds to the signals expected for a C=CH₂ unit. One plausible mechanism to obtain such compounds is presented in Scheme 27 as cyclisation mechanism A.



Scheme 27. Possible cyclisation mechanisms A and B to account for observed unexpected products.

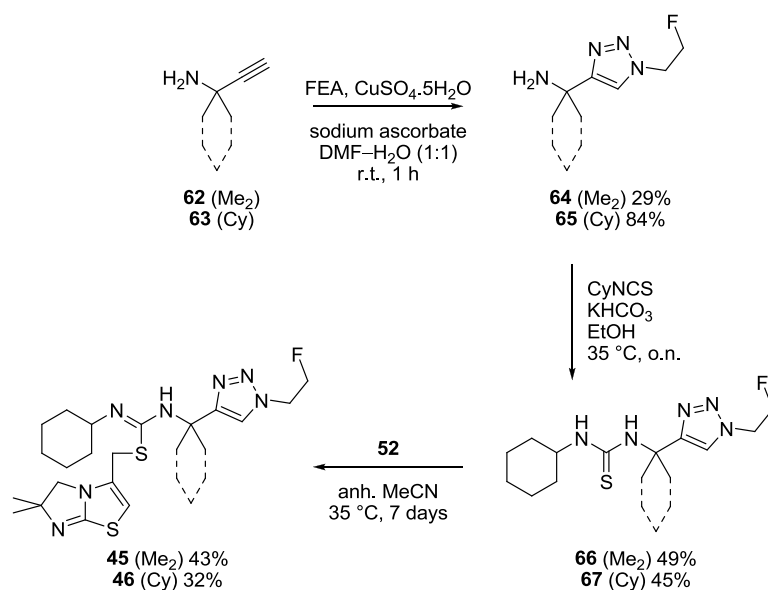
We speculated that the thiourea formed by the reaction of amine and isothiocyanate undergoes 5-*exo*-dig cyclisation involving the electrophilic alkyne to afford a 4,5-dihydrothiazol-2-amine derivative. Although this was unexpected, similar reactions have been reported in the literature,¹⁶⁰ supporting our hypothesis. The addition of NEt₃ to the reaction mixture (entry 3) minimises the formation of this by-product, which was thought to be catalysed by the presence of acid; the reaction still yielded the cyclisation product A. A change of solvent to EtOH (entry 4) was designed to provide opportunity for hydrogen bond formation between the intermediate thiourea's sulfur and the N–H in order to reduce its propensity to cyclise. Inspection of the ¹H NMR spectrum of the crude material confirmed our approach, showing only minimal C=CH₂ signal, however only the cyclised compound was obtained after flash column chromatography on silica. The reaction was probably catalysed by the acidity of silica. Substituting silica for basic alumina (entry 5) or silica basified with NEt₃ (entry 6) made no difference whatsoever. Finally, we attempted to circumvent the problem by carrying out the second step (reaction with chloride **52**) with the crude thiourea (entries 7–8), thus avoiding evaporation of EtOH. This approach appeared to protect the thiourea from cyclising presumably by avoiding contact with acidic silica. After allowing the reaction to stir for one week, the mixture was purified by preparative UV-HPLC. Two fractions were collected: one was the cyclised product A, the other was not the desired product **45** or **46**. All signals in the

^1H NMR spectrum were as expected for the desired products, however no acetylenic signal was detected. Instead, similar signals to those described above (AB system at $\delta = 5.74$ and 5.65 ppm ($J = 3.2$ Hz) with roof effect, coupling to a triplet ^{13}C at $\delta = 109.6$ ppm) made us conclude that another type of cyclisation involving the alkyne had occurred. A plausible mechanism is depicted in Scheme 27 as cyclisation mechanism B. In this putative mechanism, the resulting thiourea reacts with chloride **52** to afford the desired IT1t analogue, which in turn undergoes cyclisation in a 5-*exo-dig* fashion to produce the 4,5-dihydro-1*H*-imidazole-2-thiol.

We could not afford to place any further effort in the investigation of this reaction before having evidence that the corresponding reference compounds showed interesting preliminary activity in the binding assay.

2.2.2. *Synthesis of the reference compounds*

We anticipated that this could be achieved by reacting alkynes **62** and **63** with FEA using a click reaction, allowing us at the same time to eliminate the cyclising acetylenic functionality. Following a retrosynthetic analysis analogous to that presented in Scheme 26, alkynes **62** and **63** were cyclised with FEA in a copper(II) – sodium ascorbate system in sufficient to very good yields (Scheme 28).

Scheme 28. Synthesis of reference compounds **45** and **46**.

Formation of the thioureas on the other hand needed optimisation. Table 16 summarises the reaction conditions that were explored.

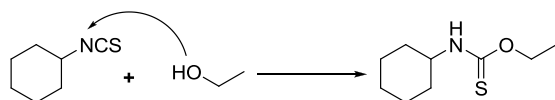
Table 16. Attempts at forming thioureas **66** and **67**.

Entry	Starting material	Solvent	Base	Reaction temperature	Reaction duration	Outcome
1	64	EtOH	K_2CO_3	$85\text{ }^\circ\text{C}$ ^a	6 h	Side-reaction A ^b
2	65	anh. MeCN	K_2CO_3	$85\text{ }^\circ\text{C}$ ^a	6 h	Side-reaction B ^b
3	64	EtOH	KHCO_3	r.t.	o.n.	Little conversion
4	65	EtOH	KHCO_3	r.t.	o.n.	Little conversion
5	64	EtOH	KHCO_3	$35\text{ }^\circ\text{C}$	o.n.	66
6	65	EtOH	KHCO_3	$35\text{ }^\circ\text{C}$	o.n.	67

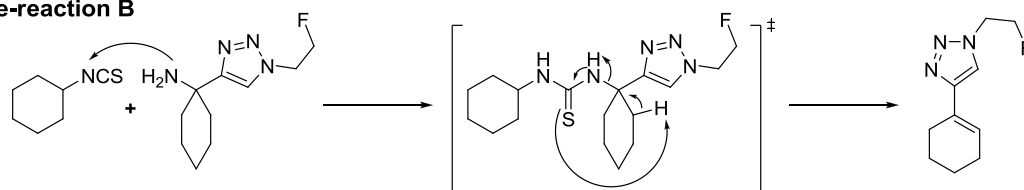
^a use of a pressure vial. ^b see Scheme 29.

We first looked at the reaction conditions employed in the synthesis of thiourea **59**. Amine **64** was reacted with CyNCS and K_2CO_3 at 85 °C in a pressure vial (entry 1). However, this resulted in the isolation of a side-reaction product as depicted in Scheme 29.

Side-reaction A



Side-reaction B



Scheme 29. Proposed mechanisms of side-reactions encountered during attempted thiourea synthesis (A and B).

The choice of EtOH as a solvent was discussed; in the presence of basic K_2CO_3 it can readily react with CyNCS to produce the corresponding thiocarbamate.¹⁶¹ This side-reaction was not observed during the formation of **59**, which suggests that the more reactive and less hindered amines would react preferentially over EtOH. However, EtOH is more likely to react with CyNCS over hindered and less reactive amine **64**.

To prevent this, we first decided to use anhydrous MeCN (entry 2) as a solvent. To our surprise, this produced yet another molecule, the structure of which was determined by ^1H , ^{13}C and ^{19}F NMR spectroscopy and is depicted in Scheme 29 as side-reaction B. Similar reactions with a substrate bearing an ester in place of the triazole was recently reported in the literature.¹⁶² This side-reaction can be rationalised thus: once formed, the thiourea, potentially assisted by K_2CO_3 , abstracts a proton from the cyclohexyl group. This generates an elimination process (type E_i) with regioselective formation of the conjugated alkene and elimination of cyclohexylthiourea.

In order to avoid this, we decided to reduce the reaction temperature and the strength of the base. When performed with $KHCO_3$ in EtOH at room temperature, although the reaction showed little conversion (entries 3–4), to our delight no by-product was observed in the

reaction mixture. Finally, warming the reaction mixture to 35 °C afforded the desired thioureas **66** and **67** overnight in moderate yields (entries 5–6).

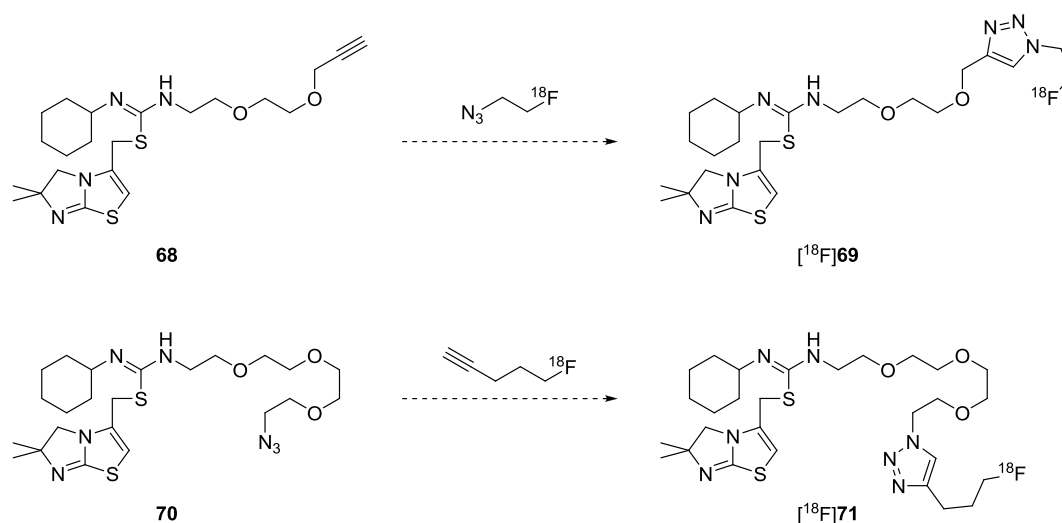
In the final step, these thioureas were successfully treated with chloride **52** to yield IT1t analogues **45** and **46** in 32–43% yield after preparative HPLC purification.

3. Expansion to a small library

3.1. Cyclisation-stable "clickable" precursors and corresponding reference compounds

3.1.1. Design

The challenge to access alkyne-containing precursors **43** and **44** prompted modification to the design of the desired precursors in order to circumvent the unwanted cyclisations encountered. Two approaches were envisaged (Scheme 30).



Scheme 30. Two new approaches for the design of "clickable" precursors.

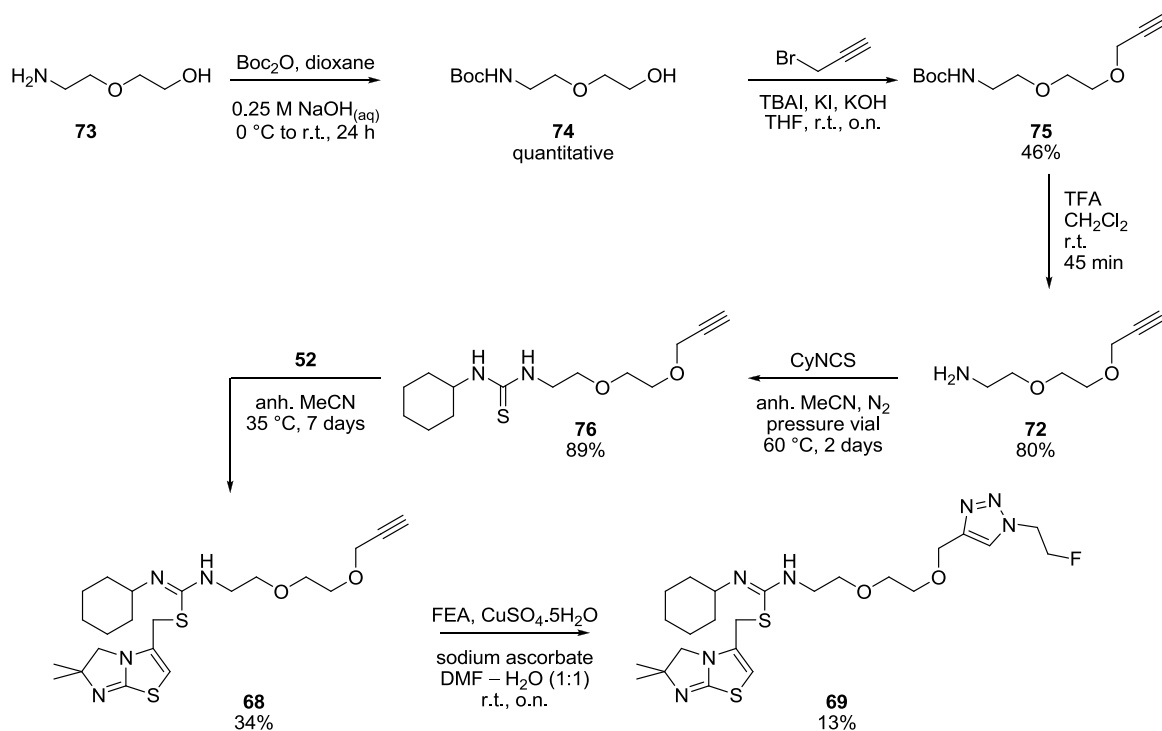
We first considered that we could modulate the reaction rate of the cyclisation of the alkyne-containing thiourea by moving the reactive functionalities further from one another. To this end, we designed analogue **68**, in which a PEG₂ spacer is used to separate the acetylene from the isothiourea functionality. This should allow us to synthesise this molecule by following a retrosynthetic analysis analogous to that for analogues **43** and **44** (Scheme 26) and avoid close

proximity between the thiourea and the alkyne reactive groups in the thiourea intermediate. Analogue **68** could then react by click chemistry with FEA to afford analogue **69**.

Second, it is possible to carry out a click reaction between an azide-bearing precursor and a fluorine-labelled alkyne. For this reason, we conceived analogue **70**, which could be labelled with 5- ^{18}F fluoropent-1-yne (**40**).⁷⁵

3.1.2. Synthesis of an alkyne-bearing precursor and its corresponding reference compound

The synthetic route to precursor **68** is analogous to that described for compounds **43** and **44**. The amine **72** used could be synthesised in three steps from commercially available aminoalcohol **73** (Scheme 31).



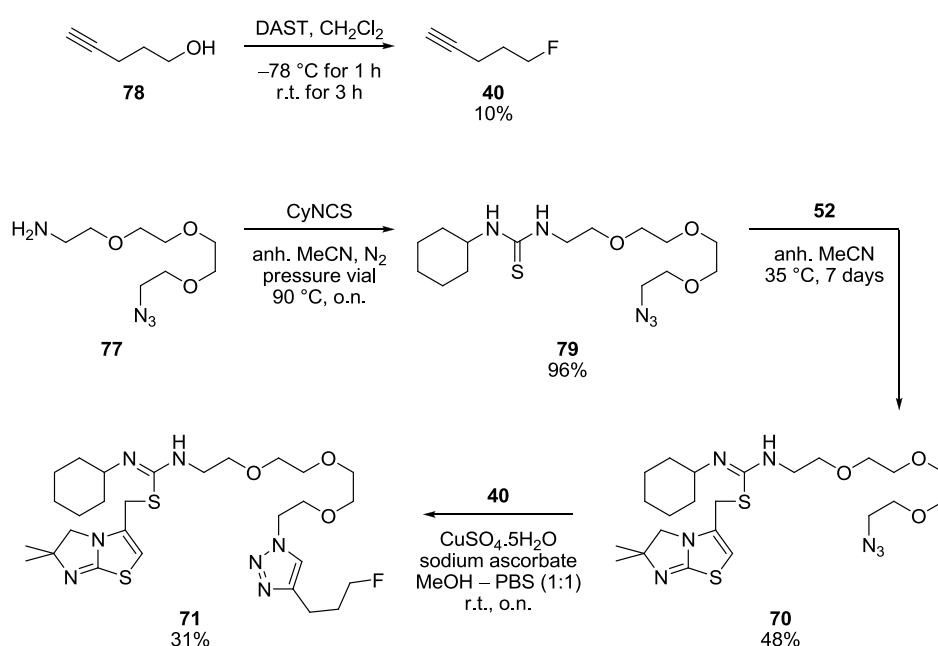
Scheme 31. Synthesis of analogues **68** and **69**.

Aminoalcohol **73** was quantitatively *N*-protected with Boc_2O before undergoing Williamson etherification with propargyl bromide followed by removal of the protecting group by treatment with TFA to afford amine **72** in very good yield. This amine was treated with CyNCS , producing alkyne-containing thiourea **76**, which was stable to flash column

chromatography on silica, thus validating our approach. Reaction of thiourea **76** with chloride **52** gave the alkyne-containing precursor **68** after preparative HPLC. This in turn underwent cyclisation with FEA by click chemistry to give the corresponding reference compound **69** as the TFA salt.

3.1.3. Synthesis of an azide-bearing precursor and its corresponding reference compound

In parallel, synthesis of IT1t analogues **70** and **71** was undertaken starting from commercially available amine **77** (Scheme 32).



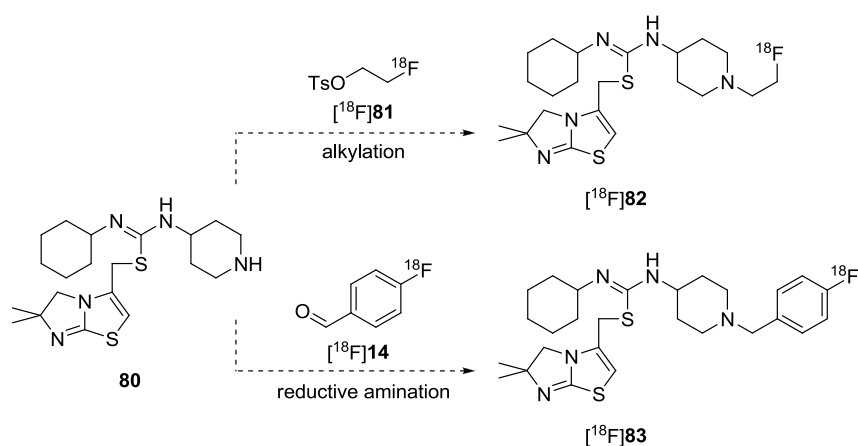
Scheme 32. Synthesis of 5-fluoropent-1-yne (**40**) and analogues **70** and **71**.

Reaction of amine **77** and CyNCS produced thiourea **79** in excellent yield, which in turn was treated with chloride **52** to afford the azide-containing precursor **70** in moderate yield. 5-Fluoropent-1-yne (**40**), synthesised by deoxyfluorination of alcohol **78** with DAST followed by distillation with a Kugelrohr apparatus, underwent [3+2]-cycloaddition with precursor **70** to yield reference compound **71** as the TFA salt.

3.2. Piperidinyl precursor and corresponding fluoroethylation and reductive elimination reference compounds

3.2.1. Design

Diversification of the labelling technologies applicable to IT1t analogues beyond the previously described click labelling strategy was sought. It is for instance possible to perform fluoroalkylation or reductive amination to label amine-containing precursors. It can be anticipated that the presence of an amine group in the tracers' structure might increase their binding affinities with CXCR4's negatively charged binding site. To this end, we designed precursor **80**, which includes a piperidine heterocycle (Scheme 33).

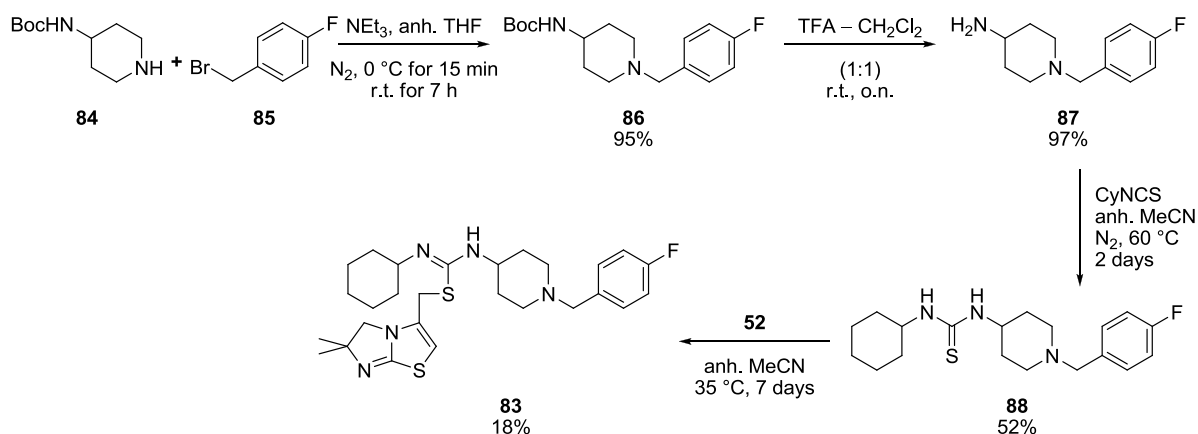


Scheme 33. Proposed piperidine-containing analogue **80**, its alkylation with tosylate $[\text{}^{18}\text{F}]$ **81** and its reductive amination with $[\text{}^{18}\text{F}]$ fluorobenzaldehyde $[\text{}^{18}\text{F}]$ **14**.

It is expected that this analogue could be reacted with 2- $[\text{}^{18}\text{F}]$ fluoroethyl tosylate ($[\text{}^{18}\text{F}]$ **81**), obtained by nucleophilic attack of $[\text{}^{18}\text{F}]$ fluoride on glycol ditosylate, to produce tracer $[\text{}^{18}\text{F}]$ **82**. Alternatively, amine **80** could undergo reductive amination by reaction with p - $[\text{}^{18}\text{F}]$ fluorobenzaldehyde ($[\text{}^{18}\text{F}]$ **14**), which could be obtained by nucleophilic aromatic substitution of $[\text{}^{18}\text{F}]$ fluoride on p -trimethylammonium benzaldehyde triflate) to produce tracer $[\text{}^{18}\text{F}]$ **83**.

3.2.2. Synthesis of the fluorobenzyl reference compound

Although the piperidine's nitrogen is expected to be the most reactive, the presence of other nitrogens in precursor **80** as well as reducible bonds would make selective reductive amination challenging. In an effort to synthesise reference compound **83** more efficiently, it was decided that the *para*-fluorotoluylyl moiety would be introduced early in the synthetic route (Scheme 34).

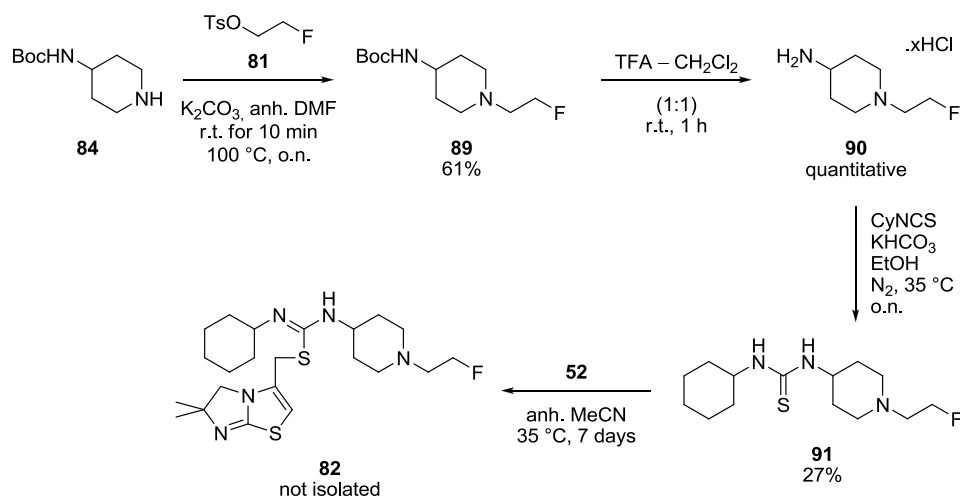


Scheme 34. Synthesis of reference compound **83**.

Thus, nucleophilic substitution of the benzylic bromide of compound **85** with the secondary nitrogen of piperidine **84** afforded in excellent yield *tert*-butyl carbamate **86**, which could be turned efficiently into the corresponding deprotected amine **87** by treatment with TFA. The amine was subsequently reacted with CyNCS, and the resulting thiourea was used to displace chloride **52** to produce reference compound **83**. This compound was converted into its hydrochloride salt with a solution of HCl in Et₂O.

3.2.3. Synthesis of the fluoroethyl reference compound

The synthetic challenges described above led us to envisage introduction of the fluoroethyl moiety early in the synthetic route (Scheme 35).

Scheme 35. First attempt at synthesising analogue **82**.

The alkylation of piperidine **84** with tosylate **81** at 100 °C afforded in moderate yield the desired product **89**, which was treated with TFA to remove the *N*-Boc protecting group. As the free amine product is rather volatile, acidification of the organic solution with a solution of HCl in Et_2O was necessary before concentration. Product **90** was obtained in quantitative yield. Nonetheless, transformation of ammonium chloride **90** into thiourea **91** necessitated some optimisation (Table 17).

Table 17. Attempts at forming thiourea **91**.

Entry	Solvent	Base	Reaction temperature	Reaction duration	Outcome
1	EtOH	K_2CO_3	90 °C ^a	6 h	Side-reaction A ^b
2	anh. MeCN	K_2CO_3	90 °C ^a	6 h	15%
3	EtOH	$KHCO_3$	35 °C	o.n.	27%

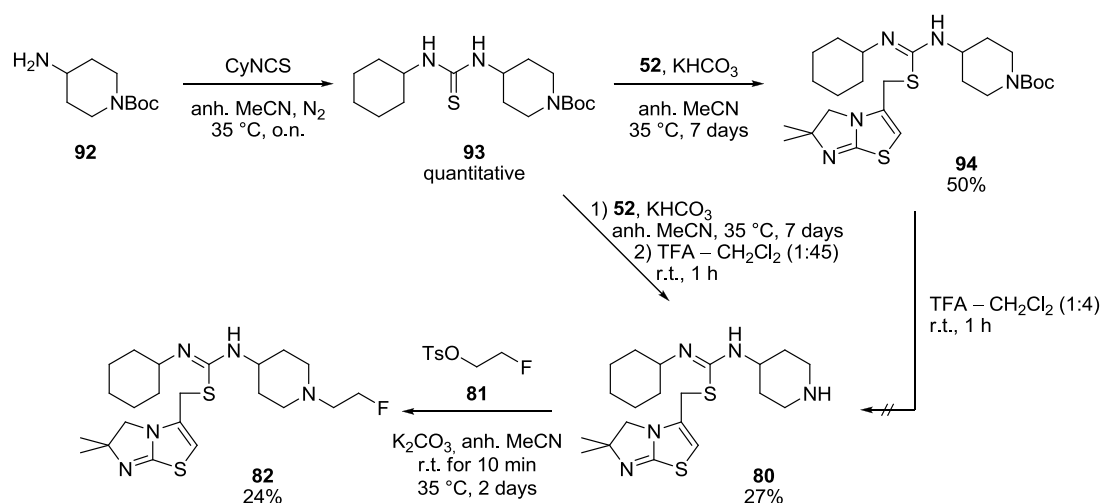
^a use of a pressure vial. ^b see Scheme 29.

As described before in Scheme 28 and Table 16, the size of amine **90** was, as for thioureas **66** and **67**, an issue. This was attributed to the competition with EtOH in the reaction with CyNCS (entry 1). Substituting EtOH for anhydrous MeCN produced the desired product in

low yield (entry 2). However, by reducing the reaction temperature to 35 °C an improved yield of 27% was obtained (entry 3). The complexity of the reaction mixture suggested the occurrence of unidentified side-reactions, which we did not consider to warrant any further optimisation. Unfortunately, subsequent reactions of thioureas and chlorides led to even poorer yields, which was probably due to presence of the tertiary amines. Although the product could be detected by high resolution mass spectrometry (HRMS) in the crude reaction mixture, various attempts at isolating the product by flash column chromatography, preparative TLC and preparative HPLC did not allow us to recover sufficient amounts of reference compound **82**.

3.2.4. Synthesis of the precursor and its corresponding fluoroethyl reference compound

Although the synthesis of reference compound **82** described above proved challenging, it was thought the introduction of a Boc protecting group would make the amine less reactive; this would in turn minimise the competing reaction (Scheme 36).



Scheme 36. Synthesis of precursor **80** and reference compound **82**.

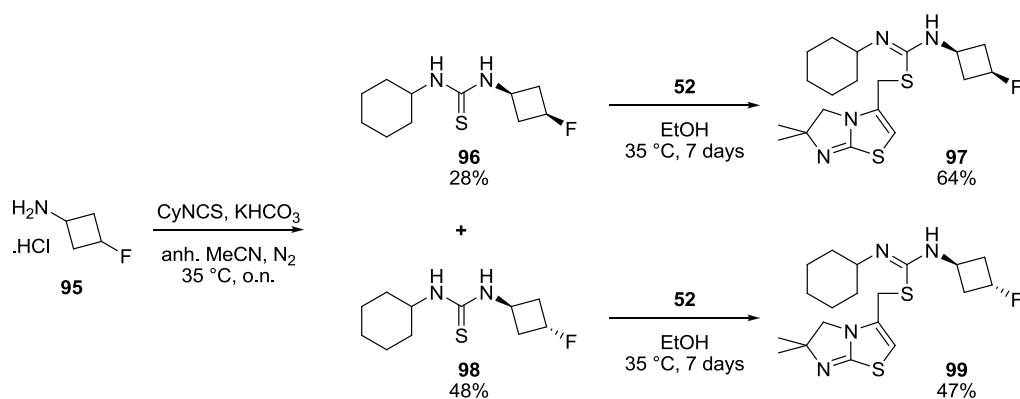
Thiourea **93** was obtained in quantitative yield from amine **92** and CyNCS , and was successfully reacted with chloride **52** to afford isothiurea **94** in 50% yield. Attempts to remove the Boc protecting group with TFA were unsuccessful. On the other hand, performing the Boc removal on the crude compound **94** afforded the desired precursor in 27% yield over

two steps. To our delight, precursor **80** reacted smoothly with 2-fluoroethyl tosylate to yield reference compound **82** in 24% yield.

3.3. Fluorocyclobutyl analogues

The challenges to synthesise our 4-fluorocyclohexyl IT1t analogue **42** (see section 2.1) did not quench our desire to access an IT1t-based tracer by direct fluorination and led to the design of two new analogues that would circumvent the elimination previously observed. Modifying the cyclohexyl moiety of the molecule to a cyclobutyl moiety should allow the fluorination to occur. This was pioneered in the field of radiochemistry by Shoup and Goodman¹⁶³ who presented an 18% end-of-bombardment RCY for the labelling of the corresponding triflate with [¹⁸F]fluoride. In more recent work,¹⁶⁴ they obtained 52% DC-RCY for the same reaction. They also achieved deoxyfluorination with DAST on the corresponding alcohol in 44% yield. The use of a tosylate leaving group instead of the triflate can also be found in the literature.¹⁶⁵ The strain of the resulting cyclobutene can account for the superior stability of cyclobutyl derivatives, making them more prone to undergo nucleophilic substitution than elimination.

The synthesis of the reference compounds was achieved as follows (Scheme 37).



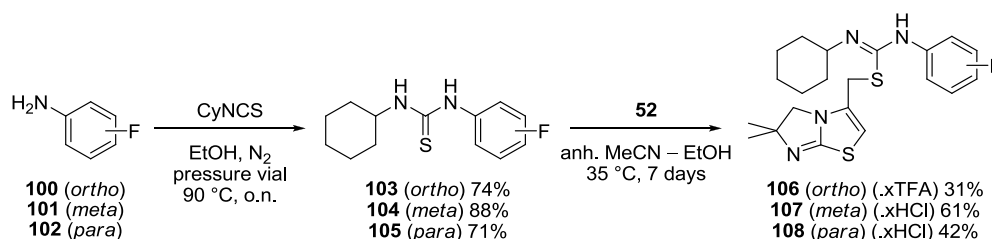
Scheme 37. Synthesis of reference compounds **97** and **99**.

Racemic ammonium chloride **95** was reacted with CyNCS to afford a mixture of the two diastereomeric thioureas **96** and **98**. The isomers were easily separated by flash column chromatography with yields of 28% and 48%, respectively. The isomers were identified by

comparison of the coupling constants found for the signal at $\delta \approx 2.70$ ppm in their ^1H NMR spectra ($J = 56.1, 13.4, 6.6, 6.6$ Hz vs. $J = 56.5, 9.8, 6.6, 3.5$ Hz), the strongest coupling being attributed to the equatorial–equatorial coupling in the *cis* isomer. Finally, the syntheses of both reference compounds **97** and **99** were achieved by reaction of the corresponding thioureas with chloride **52** in 64% and 47% yields, respectively.

3.4. Fluorophenyl analogues

Up until recently, the obtention of [^{18}F]fluorinated aromatic compounds was limited to $\text{S}_{\text{N}}\text{Ar}$ with electron-poor rings or the use of carrier-added [^{18}F]fluoronium species with electron-rich rings. In 2011, Ritter *et al.* published a ground-breaking method to directly label aromatic groups with [^{18}F]fluoride *via* a palladium-catalysed reaction.⁸⁷ Recently, improvement of this work led to the use of aqueous [^{18}F]fluoride.⁸⁸ Inspired by this work, we devised three other IT1t analogues that could be radiosynthesised using this new technology (Scheme 38).



Scheme 38. Synthesis of reference compounds **106**, **107** and **108**.

Starting from the *ortho*-, *meta*- and *para*-fluoroaniline series (**100**, **101**, **102**), the corresponding thioureas were obtained by reaction with CyNCS in good to very good yields. Subsequently, the resulting thioureas **103**, **104** and **105** were reacted with chloride **52** to afford the desired analogues **106**, **107** and **108**, respectively. Whereas the *meta*- and the *para*-analogues readily precipitated from the reaction mixture as dihydrochloride salts in analytically pure form, the *ortho*-analogue did not and required preparative HPLC purification.

3.5. Gallium-68-labellable analogues

3.5.1. Design

Finally, to complete our collection of potential PET tracers based on the structure of IT1t, we investigated the possibility to employ gallium-labelling in such a small molecule. It was anticipated that the size of the chelating group would be the major challenge for this type of tracer. Thus, because the binding site within CXCR4 is rather deep, steric hindrance between the chelating group and the receptors was expected. In order to prevent this, the introduction of a spacer PEG unit was envisaged. Nonetheless, the optimal size of the spacer was to be determined.

To this end, we designed and docked *in silico* 32 candidate PET tracers into CXCR4's binding site. This was with a view to address three key questions: the nature of the spacer unit, the length of the spacer unit and the optimal chelating group (Figure 43).

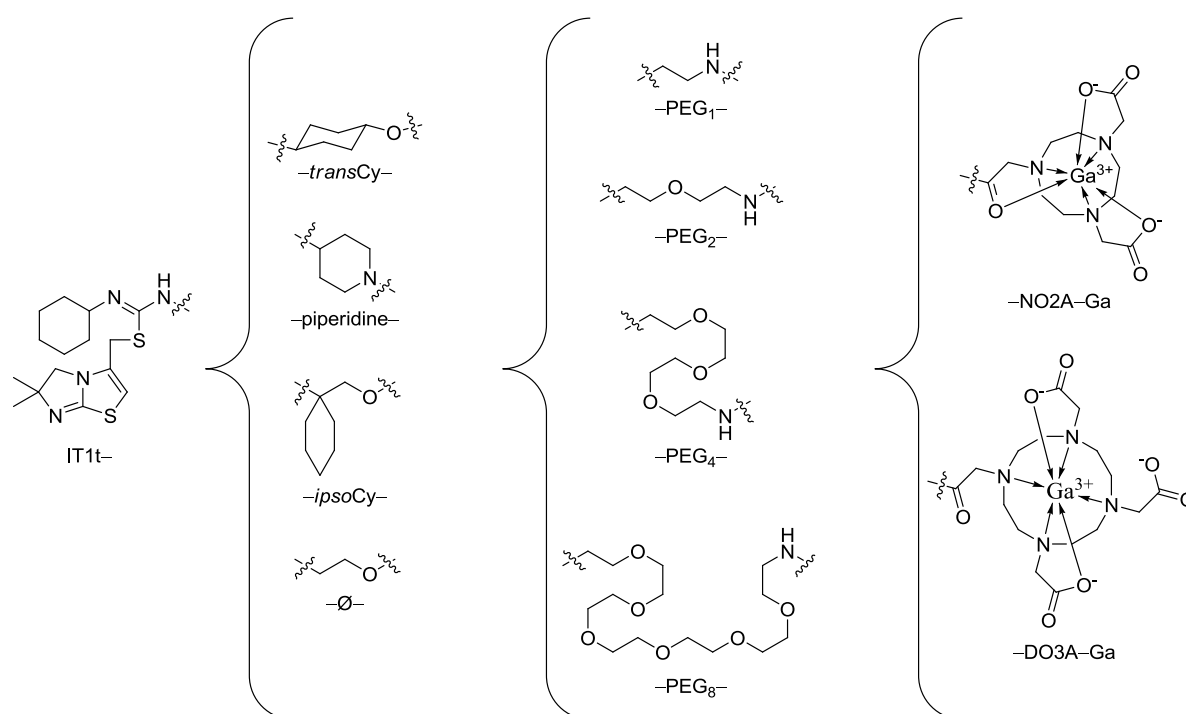


Figure 43. Structure of the 32 analogues designed to be docked *in silico* into CXCR4's binding site.

We explored all combinations of the building blocks presented in Figure 43. Keeping the IT1t core constant (IT1t-), we investigated adding different linking units to probe potential

additional interactions with CXCR4. Introduction of *trans*-cyclohexanoxy (*–transCy–*) and piperidin-1-yl (*–piperidine–*) groups were expected to mimic the cyclohexyl group present in IT1t but also allow modification on the far side of it. The other group, the *ipso*-cyclohexylcarbinoxy (*–ipsoCy–*) group was meant to recreate a structure similar to that of our "clicked" reference compound **46** and a simple ethylene glycol unit (*–O–*) was to be investigated as a control. We then investigated four different sizes of PEG units, namely *–PEG*₁*–*, *–PEG*₂*–*, *–PEG*₄*–* and *–PEG*₈*–*. Finally, we wanted to probe the potential differences between NO2A and DO3A chelating groups.

Unfortunately, modelling a metal chelating to a ligand is not feasible using the Schrödinger software suite. However, leaving the NO2A and DO3A free of chelation would significantly alter the nature of interactions that the chelated ligand makes with CXCR4's binding site. Therefore, we decided to substitute the gallium-chelating group moieties by a carbon-based surrogate for the purpose of the *in silico* modelling (Figure 44).

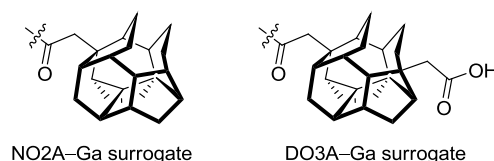


Figure 44. NO2A–Ga and DO3A–Ga surrogates used for the docking study.

We ornamented this "blob" with a pendant *–CH*₂*–CO*₂*H* group to simulate gallium-chelated DO3A's free carboxylate as found in the crystal structure of DOTA–Ga as it could have some impact on the docking score.⁹⁰

Once designed, the ligands were prepared with the LigandPrep tool (using default settings) and the structures were docked in the previously defined docking grid (see section 1.2) with Glide. Unfortunately, this yielded no conclusive data, as most of the poses were very dissimilar to that of the co-crystallised IT1t. We hypothesised that this was due to the strong differences (in particular in size) between IT1t and the docked ligands. Therefore, we introduced constraints in the grid in order to force the aromatic core of our candidate ligands to dock within a certain distance to the co-crystallised IT1t (namely, the aromatic sulfur, the benzylic methylene and the alicyclic quaternary carbon within 1 Å of their co-crystallised

counterparts). Obviously, these constraints limit the extent of the conclusions that can be drawn from this computational work. Therefore, these results were treated with greater caution than originally intended.

Limiting our analysis to the 28 best docking scores by setting a cut-off at 80% of the refined docking score of IT1t (refined IT1t: -7.83), we made four observations. First of all, out of these 28 poses, 25 contained a DO3A while 3 contained a NO2A, which suggests that the pendant carboxylic acid in DO3A–Ga moiety makes some interactions with the positively charged rim of the helix bundle. Second, the best pose was displayed by IT1t–*ipso*Cy–PEG₄–DO3A–Ga, which also counted the most poses with five single poses among the 28 short-listed, the five of which overlap markedly with the pose adopted by the co-crystallised IT1t (Figure 45).

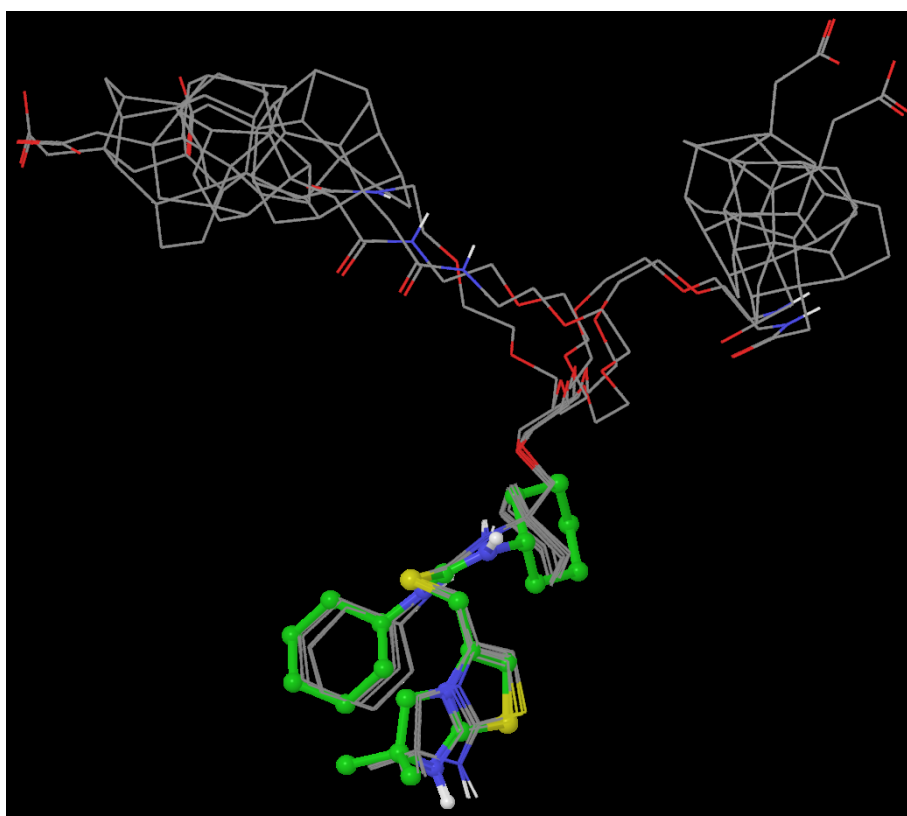


Figure 45. Overlay of IT1t (ball and stick) and of the five poses of IT1t–*ipso*Cy–PEG₄–DO3A–Ga.

In three of those poses, a salt bridge between DO3A–Ga's free carboxylate and the receptor's rim is exhibited, supporting our previous suggestion. Furthermore, the –*trans*Cy– and –

piperidine– containing candidates tended to show a distorted binding mode, with the isothiourea functional group pointing in the opposite direction and the unmodified cyclohexyl binding in a different pocket, indicating potential steric hindrance. Finally, –Ø–PEG₁– presented the shortest spacer distance between the core and the chelator but this PEG length appears to be too short for the cyclohexyl-bearing candidates which favour a –PEG₄– spacer.

Analysis of this study suggests that –DO3A–Ga shows superior characteristics to –NO2A–Ga thanks to its capacity to form a salt bridge with the positively charged rim of the receptor's helix bundle and that IT1t-*ipso*Cy–PEG₄–DO3A–Ga's poses are consistent with that of IT1t in the co-crystal structure. This analogue was therefore selected as candidate for further investigation. (Figure 46).

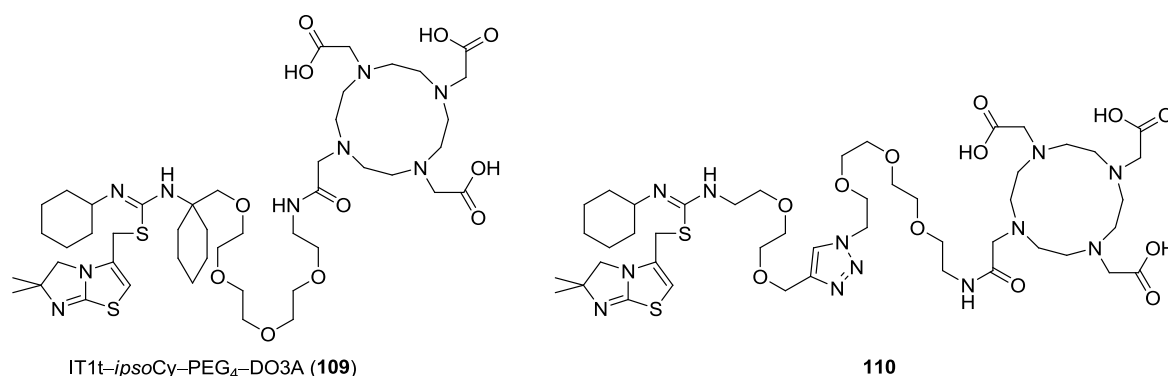
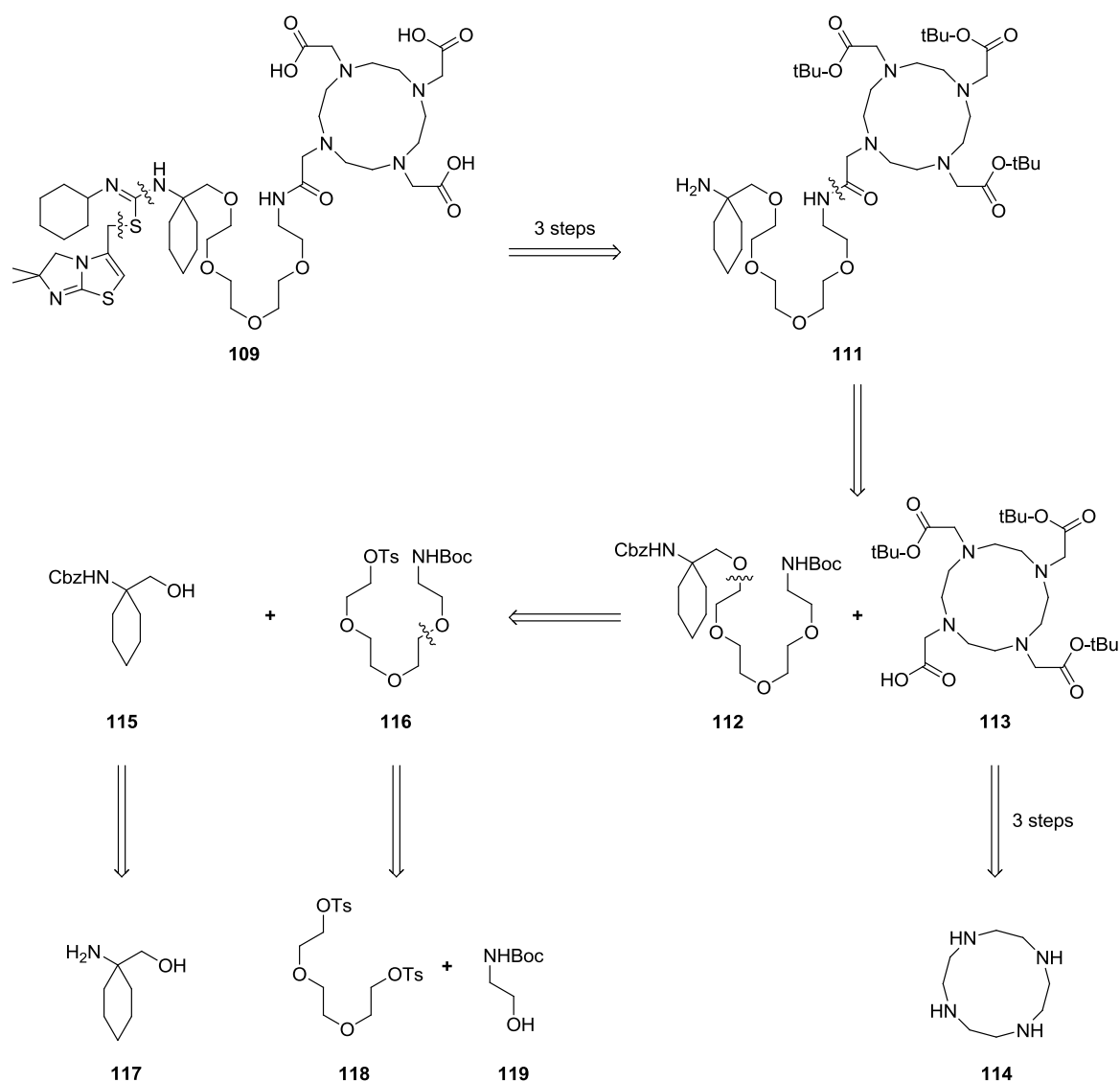


Figure 46. Proposed analogues for gallium-chelating precursors.

Moreover, although the –Ø– structures exhibited poorer docking scores, it was deemed prudent to synthesise one of them as docking studies, especially with GPCRs and more in particular because of the constraints we had to set up in order to obtain docked structure, are to be considered with caution (see section 1.2). Analogue **110**, potentially obtainable in a few steps from precursor **68** previously synthesised, was considered for synthesis.

3.5.2. Synthesis of the *ipso*-cyclohexyl precursor

Precursor **109** can be obtained from its *tris*(*O*-tBu) protected congener by treatment with acid. The resulting parent compound's IT1t core could be obtain as previously described by reaction of the corresponding amine **111** with CyNCS and chloride **52**, sequentially (Scheme 39).

Scheme 39. Retrosynthetic analysis towards precursor **109**.

Compound **111** could be synthesised by amide bond formation between the adequate amine and carboxylic acid **113**. However, this parent amine would have two amine groups, therefore necessitating orthogonal protecting groups such as Boc and Cbz, as depicted by the structure of compound **112**. The synthetic pathway to DOTA(*O*-*t*Bu)₃ (**113**) from cyclen (**114**) is well described in the literature.^{166,167} Then, compound **112** could be accessed by etherification between alcohol **115** (obtained by *N*-Cbz protection of amine **117**) and tosylate **116**. Finally, etherification between ditosylate **118** and alcohol **119** is expected to yield compound **116**.

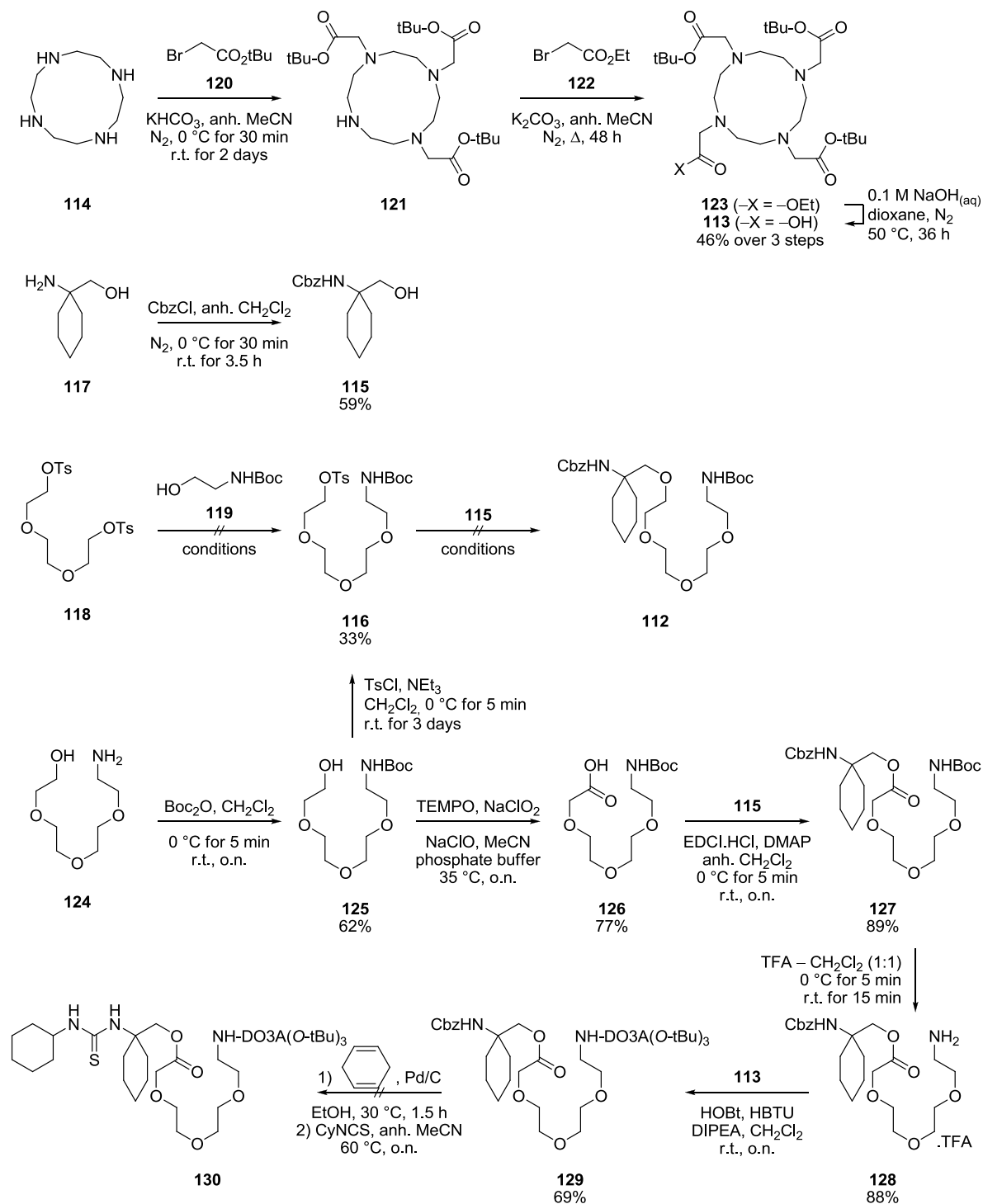
Although this synthetic pathway comprises 10 steps, we anticipated that this convergent approach would allow the desired precursor to be obtained in good yield.

The synthetic effort towards compound **109** is described below. The initial target was to synthesise carboxylic acid **113** (Scheme 40).

Reaction of *tert*-butyl bromoacetate (**120**) with cyclen (**114**) afforded triester **121**, which was purified by recrystallisation.^{166,167} This compound underwent further alkylation with bromide **122**, and the resulting ethyl ester **123** was saponified to give DOTA(*O*-*t*Bu)₃ (**113**) in an overall yield of 46% over 3 steps. In parallel, amine **117** was protected with CbzCl in moderate yield (59%). A first attempt at synthesising the –PEG₄– spacer group employed an etherification reaction between alcohol **119** and ditosylate **118** to give tosylate **116**. Two attempts using NaH, DMF, N₂, r.t., 1.5 h following literature procedure were performed,¹⁶⁸ however analysis of the crude reaction mixture by HRMS and ¹H and ¹³C NMR spectroscopy showed loss of the Boc protecting group and subsequent cyclisation. To circumvent this problem, an *N*-Boc protection and *O*-tosylation reaction sequence with aminoalcohol **124** afforded the desired tosylate **116**. Unfortunately, etherification of this compound with alcohol **115** following an alternative literature procedure¹⁶⁹ to that described previously (NaOH_(aq), toluene, 100 °C, 5 h) gave an ambiguous outcome: although HRMS did not display any molecular peak, the loss of the Cbz protecting group was apparent in the ¹H NMR spectrum. The fact that Cbz-deprotection occurred suggests it is highly possible that the resulting free amine displaced the tosylate, affording a compound that would be difficult to discriminate and to separate from the desired product **112**.

We therefore resolved to change slightly the structure of the final compound **109** to allow coupling the –*ipso*Cy– moiety with the –PEG₄– moiety by esterification. Thus, alcohol **125** was oxidised under mild conditions to the corresponding carboxylic acid by a (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) -catalysed reaction in 77% yield.¹⁷⁰ The resulting carboxylic acid was successfully coupled to alcohol **115** with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI.HCl) in very good yield. Removal of the Boc protecting group by treatment with TFA produced amine **128**. Coupling of this amine with DOTA(*O*-*t*Bu)₃ (**113**) mediated by HOBt–HBTU–DIPEA afforded the *N*-Cbz protected –*ipso*Cy–PEG₄–DO3A(*O*-*t*Bu)₃ derivative **129** in 69% yield. Although removal of the carboxybenzyl group with Pd/C and cyclohexa-1,4-diene¹⁷¹ went to completion by TLC, only little material was collected by filtration through Celite® (~30%, crude). Nonetheless, the

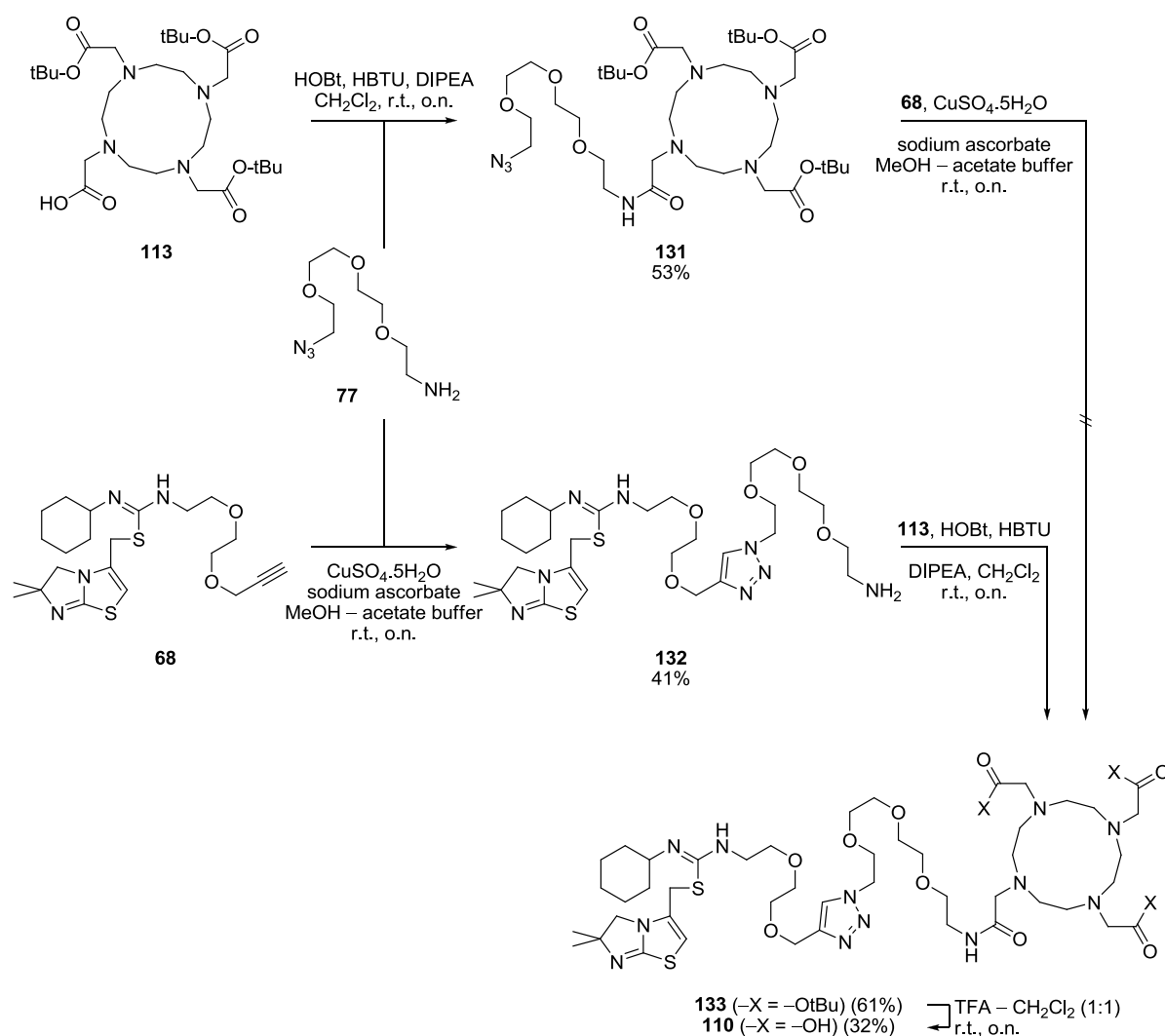
crude material was subjected to reaction with CyNCS. However, the product **130** could not be isolated either by flash column chromatography or by preparative TLC.

Scheme 40. Attempts at synthesising precursor **109**.

Eventually, we turned our attention to the synthesis of **110**, which appeared to be more tractable.

3.5.3. Synthesis of a precursor by click chemistry

Although more direct, the synthesis of this precursor by click chemistry also proved challenging (Scheme 41).



Scheme 41. Synthesis of precursor **110**.

We first envisaged synthesising precursor **110** by reacting previously described alkyne **68** with literature azide **131** under CuAAC conditions. Whereas compound **131** was readily

obtained from the amide bond formation between DOTA(*O*-tBu)₃ (**113**) and amine **77**, the click reaction afforded after preparative HPLC purification an amorphous powder bearing a distinctive blue colour, suggesting chelation of copper within the cyclen ring. This was further confirmed by HRMS and the general broadness of the peaks in the ¹H NMR spectrum. It is known from the literature⁹⁰ that copper(II) chelates with cyclen ($\log K_{\text{ML}} = 24.6$, no account on the reversibility) and also with cyclam ($\log K_{\text{ML}} = 27.2$, irreversibly). A solution of our **133**–copper chelate was mixed with cyclam. Preparative HPLC purification of this mixture and lyophilisation of the collected fraction regrettably afforded a blue powder again. Transmetallation from cyclam to cyclen could have happened in the first seconds of the HPLC purification, namely during the concentration at the entry of the column. Because of this, we attempted loading the blue material onto a Waters SepPak C18+short SPE cartridge and washed it with a very large excess of cyclam solution, however this still resulted in the obtention of a blue powder after elution off the cartridge and lyophilisation. Finally, as described later (see section 4.1.1) because of the DOTA's preference for Ga(III) ($\log K_{\text{ML}} = 23.9$) rather than Cu(II) ($\log K_{\text{ML}} = 22.2\text{--}22.7$), we carried out some radioactive work with it. Unfortunately, none of these afforded the desired tracer. Therefore, a new synthetic route to precursor **110** was devised.

This required marking a modification to amide and click reaction steps. Inverting the two steps of amide bond formation and CuAAC worked in our favour: thus, amine **132** was obtained in 41% yield after preparative HPLC purification with no copper chelated. Then, amide bond formation with DOTA(*O*-tBu)₃ (**113**) afforded the *O*-*tert*-butyl protected precursor **133** in 61% yield. Finally, conversion of the *tert*-butyl ester into the corresponding carboxylic acid produced the desired precursor **110** in modest yield and quantity (32%, 1.66 mg). The limited quantities of this compound prompted a rethink as far as reference compound was concerned. To obtain the reference compound would require saponification. However, owing to the limited amount of the precursor in hand radiolabelling of this intermediate was investigated with a view to explore *in vitro* cellular uptake.

4. Radiolabelling

4.1. Gallium-68

4.1.1. Radiolabelling of the copper-chelated precursor

As aforementioned, we attempted to carry out radiolabelling with our sample of compound **133** contaminated with copper. In the literature,⁹⁰ the stabilities of various complexes under acidic conditions have been reported. For instance, the DOTA-copper chelate has a half-life of less than 3 min in 5 M HCl at 90 °C. Unfortunately, all attempts at using this data yielded little to no formation of the desired tracer (Table 18).

The general strategy was to simultaneously perform the *tert*-butyl ester deprotection and removal of the copper under acidic conditions and then to introduce gallium-68 into the structure. Placing precursor **133** directly in the generator's eluate (0.1 N HCl) yielded limited amount of radioactive material from the desired tracer (entry 1–3). An even lower pH (entry 4–5) was detrimental to the conversion. We hypothesised that this deterioration was caused by protons competing with gallium-68 for the binding to the DOTA moiety. Therefore, it was anticipated that increasing pH to a value favourable to gallium-chelation in a second step could positively influence conversion (entry 6–7). Although it did not do so to a reasonable extent, further attempts involving the addition of a large excess (entry 8–9) or a lower amount (entry 10–11) of cyclam to the labelling mixture resulted only in the labelling of cyclam to various low degrees.

These disappointing results led us to consider alternative synthetic route to compound **110**, which would preclude copper contamination.

Table 18. Attempts at labelling the copper-chelated precursor **133** with gallium-68.

Entry	Conditions	t (min)	Conversion
1	0.1 N HCl (generator, 1 mL)	5	0.46%
2	0.1 N HCl (generator, 1 mL)	20	1.0%
3	0.1 N HCl (generator, 1 mL)	60	1.9%
4	1) 100 μ L conc. HCl (5 min) 2) 0.1 N HCl (generator, 1 mL)	5	0.0%
5	1) 100 μ L conc. HCl (5 min) 2) 0.1 N HCl (generator, 1 mL)	20	0.0%
6	1) 0.1 N HCl (generator, 1 mL, 5 min) 2) 1 mL NaAB ^a	5	2.4%
7	1) 0.1 N HCl (generator, 1 mL, 5 min) 2) 1 mL NaAB ^a	20	4.7%
8	1) 0.1 N HCl (generator, 1 mL, 5 min) 2) cyclam (1 mg in 1 mL NaAB ^a)	5	cyclam labelling (95%)
9	1) 0.1 N HCl (generator, 1 mL, 5 min) 2) cyclam (1 mg in 1 mL NaAB ^a)	20	cyclam labelling (97%)
10	1) 0.1 N HCl (generator, 1 mL, 5 min) 2) cyclam (25 μ g in 1 mL NaAB ^a)	5	cyclam labelling (22%)
11	1) 0.1 N HCl (generator, 1 mL, 5 min) 2) cyclam (25 μ g in 1 mL NaAB ^a)	20	cyclam labelling (46%)

All experiments were carried out at 90 °C, with 50 μ L of a solution of compound **133** in MeOH (1.0 mg.mL⁻¹). Reaction duration (t) was measured from the last stage described in the conditions.

^a sodium acetate buffer, pH 5.8, 0.5 M.

4.1.2. Radiolabelling of the non-copper-chelated precursor

Optimisation of precursor **110**'s labelling with gallium-68 was carried out with various concentrations of precursor, using 1 mL of the generator's eluate and following the same procedure as devised for the labelling of our T140 analogue, namely: sodium acetate buffer (pH 5.8, 0.5 M), 90 °C (Table 19).

Table 19. Optimising the labelling of precursor **110**, at 5, 10 and 15 min reaction duration for various precursor concentrations. Data are mean $\pm$ SD of four independent experiments or range of two independent experiments.

Reaction duration	Precursor concentration			
	0.2 mg.mL ⁻¹	0.5 mg.mL ⁻¹	1.0 mg.mL ⁻¹	2.0 mg.mL ⁻¹
5 min	0 – 5.7%	32 – 38%	48 $\pm$ 13% (<i>n</i> = 4)	94 – 95%
10 min	–	–	54 $\pm$ 5% (<i>n</i> = 4)	–
15 min	0 – 6.0%	38 – 40%	93 $\pm$ 2% (<i>n</i> = 4)	99%

As anticipated, the gallium-labelling conditions (1.0 mg.mL⁻¹) devised for our T140 analogue, **21**, applied very well to precursor **110**. In the attempt to optimise the conditions, we could observe that labelling expectedly increased with reaction duration and precursor concentration. Reducing the amount of precursor in the reaction vial in order to enhance specific activity of the delivered material did not provide a rapid enough conversion rate. On the other hand, increasing the amount of precursor to 2 mg.mL⁻¹ yielded nearly full conversion within 5 min. Nevertheless, we decided to keep the original procedure to carry out the full scale labelling as a balance between labelling duration and specific activity.

The full-scale experiments were consistent with the previous experiments.

Table 20. Labelling of precursor **110**.

Entry	Conversion at 15 min	Radiochemical purity	Apparent specific activity (GBq.μmol ⁻¹)	Duration	RCY
1	86%	100%	–	24 min	17%
2	89%	100%	1.28	22 min	27%
3	94%	100%	1.22	24 min	31%
4	93%	100%	1.58	24 min	26%
5	95%	99%	1.11	25 min	24%
6	–	96%	1.55	22 min	33%
7	–	100%	1.38	20 min	30%

They showed an excellent conversion ($91\pm4\%$, $n = 5$), affording the tracer in excellent purity ($99\pm1\%$, $n = 7$) and satisfactory specific activity (1.4 ± 0.2 GBq. μmol^{-1} , $n = 6$). The non-decay corrected yield was $27\pm5\%$ ($n = 7$) and the whole delivery process took a total of 23 ± 2 min ($n = 7$).

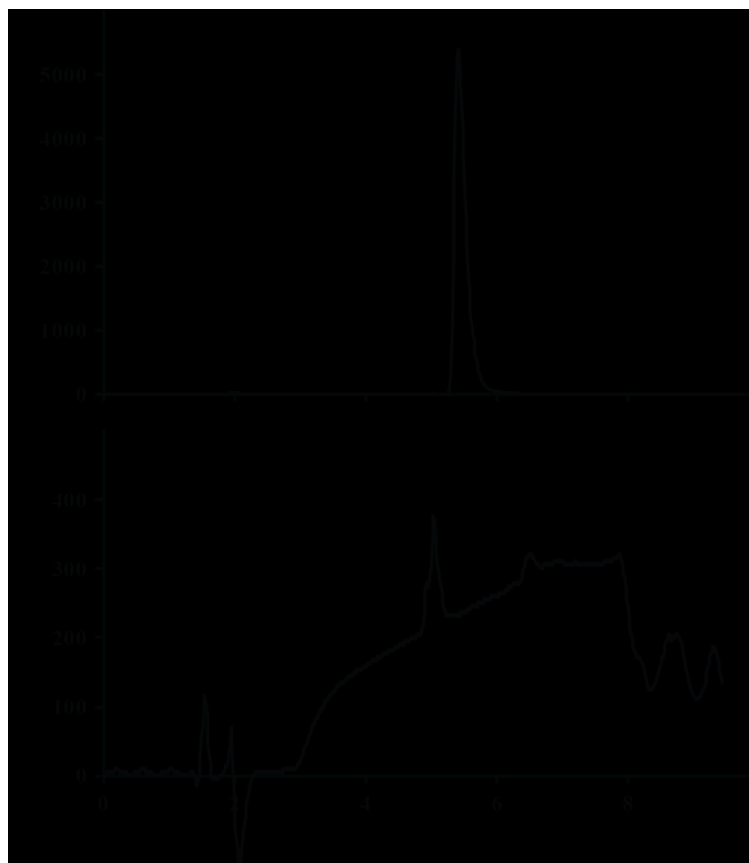


Figure 47. Quality control of the delivered gallium-68-labelled **110**. A: radioactive trace of the formulated tracer. B: UV chromatogram of the formulated tracer, $\lambda = 210$ nm.

4.2. Fluorine-18-labelling of the cyclisation-stable "clickable" precursor

4.2.1. Description of the procedure

For the radiosynthesis of compound **69**, we chose to follow the procedure developed by Årstad *et al.*¹⁷² In this procedure, [^{18}F]FEA is formed as described by Glaser and Årstad⁷⁶ and in contrast to the protocol used previously with our cyclopentapeptides (see section III.2.2) the produced [^{18}F]FEA was not distilled, but the reaction mixture was used crude for the labelling step in a one-pot fashion. As indicated earlier, the labelling step is performed

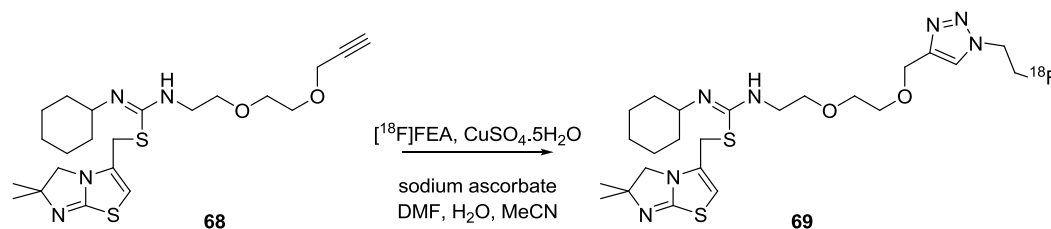
between the radioactive azide and an alkyne-containing precursor with CuSO_4 , sodium ascorbate and bathophenanthrolinedisulfonic acid disodium salt hydrate (**30**). As the resulting reaction mixture consists of crude material in quantities unsuitable for semi-preparative HPLC (*e.g.*, $\sim 30 \mu\text{mol}$ KHCO_3 or K_2CO_3 , $15 \mu\text{mol}$ $\text{K}_{2.2.2}$, $8.4 \mu\text{mol}$ unreacted 2-azidoethyl tosylate and by-products thereof, $10 \mu\text{mol}$ unreacted alkyne precursor and by-products thereof, $8 \mu\text{mol}$ copper, $40 \mu\text{mol}$ sodium ascorbate and $8 \mu\text{mol}$ ligand **30**), a pre-purification step is required. This is achieved by solid-phase extraction, preferentially with a C18 coated cartridge. This allows separation of on one side polar material (carbonate, $\text{K}_{2.2.2}$, sodium ascorbate, copper) and unreacted $[\text{}^{18}\text{F}]\text{FEA}$ (which is too small to be retained), on the other side mildly polar material ($[\text{}^{18}\text{F}]\text{tracer}$, precursor, unreacted 2-azidoethyl tosylate) and leave on the cartridge ligand **30**. The fraction containing the tracer can therefore be purified by semi-preparative HPLC, and the collected HPLC fraction is reformulated with EtOH–PBS.

Expectedly, using an isocratic HPLC elution method (20% B) allowed good separation between the precursor and the tracer, which entails that no scavenging step is necessary for this tracer.

4.2.2. Development

All radioactive work was carried out manually and as described above. Our first attempt at labelling precursor **68** proved extremely suboptimal (Table 21).

Table 21. Optimisation of the labelling procedure for precursor **68**.



Entry	Volume tosylate mixture (μL)	Waters C18 Cartridge	Additive ^e	Reaction mixture dilution (mL)	Cartridge trapping (%) ^d	Washed out (%) ^d	Eluent (% MeCN–H ₂ O)	Additive ^e	Eluate ^f	Cartridge (still trapped) ^f
1	400	light	none	2	11	89	1 mL 20%	none	5	95
2	400	light	none	10	32	68	1 mL 30%	none	7	93
3 ^a	–	–	–	–	–	–	+ 1 mL 50%	none	40	60
4 ^a	–	–	–	–	–	–	+ 0.5 mL 50%	HCl	89	11
5	400	light	TFA	10	41	59	0.5 mL 50%	TFA	51	49
6 ^a	–	–	–	–	–	–	+ 0.5 mL 50%	TFA	88	12
7	200	light	TFA	10	26	74	0.5 mL 50%	TFA	55	45
8 ^b	200	light	TFA	10	59	41	1 mL 50%	TFA	82	18
9 ^b	200	plus	TFA	10	46	54	2 mL 50%	TFA	49	51
10 ^b	200	2 light	TFA	10	79	21	2 mL 50%	TFA	90	10
11 ^b	200	2 light	TFA	10	80	20	2 mL 50%	TFA	89	11

^a Additional elution of the same cartridge as above. ^b [¹⁸F]Fluoride dried with additional care.

^c Additive used for the cartridge preconditioning and reaction mixture dilution (TFA: 0.1%).

^d Represents the fraction of radioactivity compared to the amount injected in the cartridge. ^e Additive used for the cartridge elution (TFA: 0.1%; HCl: 0.1 N). ^f Represents the fraction of radioactivity compared to the amount trapped in the cartridge.

Only 11% of the radioactivity contained in the reaction mixture was trapped on the Waters Light C18 cartridge, and only 5% of it was eluted by 20% MeCN–H₂O (entry 1). These two parameters therefore needed to be optimised. Reducing 5-fold the organic content of the mixture injected into the cartridge increased the trapping rate by a factor of 3 (entry 2). Besides, increasing the eluent strength to 50% MeCN–H₂O slightly increased the recovery rate (entry 1–3), but in no comparison with acidifying the eluent: 89% of the activity on the cartridge was released this way (entry 4). With this in mind, we decided to work with TFA as

an additive from the cartridge preconditioning to the elution of the tracer, since TFA is the additive used for the semi-preparative HPLC in the next stage. This slightly improved the trapping, but above all ameliorated the cartridge release to 88% (entry 5–6). Analysing the origin of the radioactivity washed out of the cartridge when loading it, we noticed that it contained mostly [^{18}F]fluoride and [^{18}F]FEA, meaning that neither the displacement of the tosylate nor the cycloaddition reaction are brought to completion in our conditions. Reducing the reaction mixture's volume (entry 7) did not improve the overall RCY, but brought the cycloaddition reaction to completion after the HPLC trace of the washed out radioactivity sample. Drying [^{18}F]fluoride to a greater extent (entry 8) resulted in nearly full conversion of [^{18}F]fluoride into the tracer, and 59% of the reaction mixture's radioactivity was trapped on a Waters Light C18. Using a larger Waters Plus C18 aggravated further the trapping and reduced the amount of radioactivity released from the cartridge by nearly half (entry 9). On the other hand, using two Waters Light C18 cartridges in series boosted the radioactivity trapping to 80%, 90% of which were released with 50% MeCN–H₂O + 0.1% TFA reproducibly (entry 10–11).

4.2.3. Delivery statistics

The resulting eluate was diluted with H₂O + 0.1% TFA and successfully purified by semi-preparative HPLC and reformulated in 10×100 μL EtOH.

Overall, our tracer [^{18}F]**69** was obtained in 33±13% DC-RCY in 40±26 min from [^{18}F]FEA and in 18±6% EOS-RCY in 85±24 min from aqueous [^{18}F]fluoride, starting with up to 1.1 GBq, in >99% radiochemical purity ($n = 3$) (Figure 48).

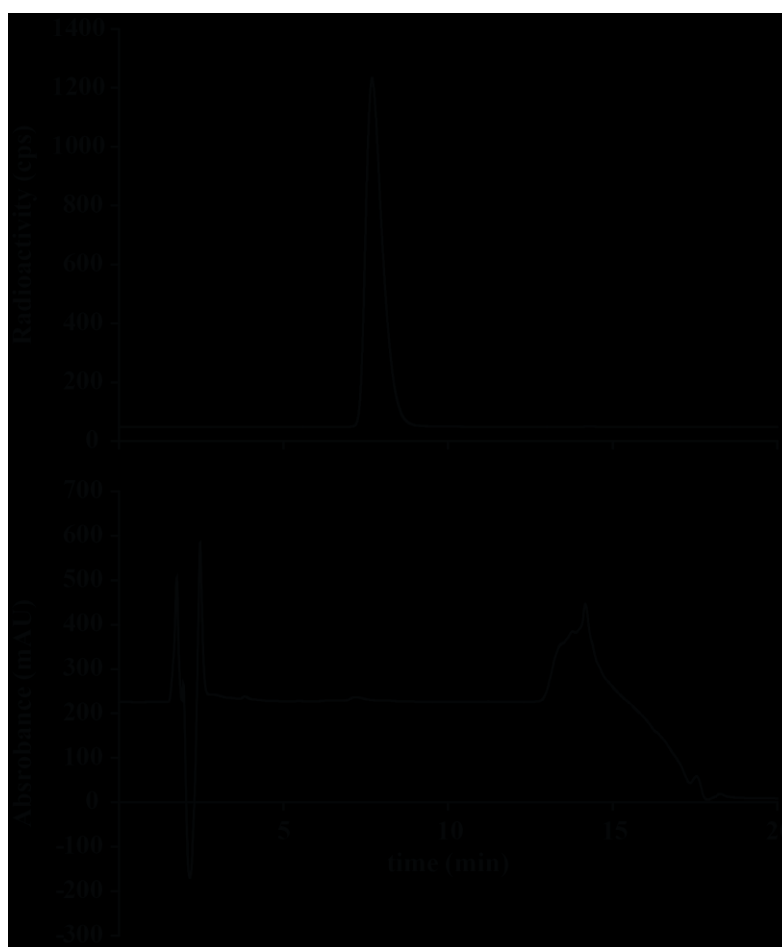


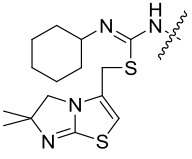
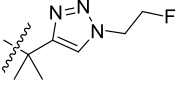
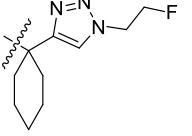
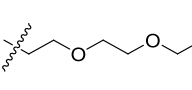
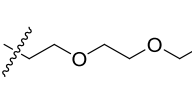
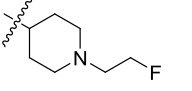
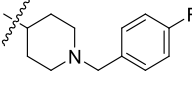
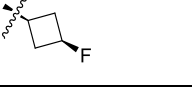
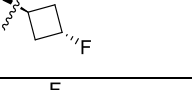
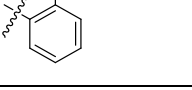
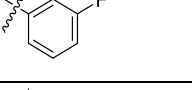
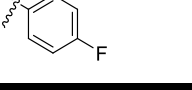
Figure 48. Quality control of the delivered tracer [^{18}F]**69**. A: radioactive trace of the formulated tracer. B: UV chromatogram of the formulated tracer, $\lambda = 210$ nm.

5. Biological results

5.1. Binding and migration assays

The antagonistic activities of synthesised IT1t analogues were assessed *in vitro* by radioligand binding and migration assays (Table 22).

Table 22. CXCR4 binding affinities of IT1t analogues in radioligand binding and migration assays.

Entry	Compound	Structure	IC ₅₀ (μM)	
			Binding	Migration
1	IT1t		0.0015±0.0004	0.0521±0.0026
2	45		>50^a	>100
3	46		>5^a	27.7±9.14
4	69		>100	>100
5	71		n.d.	n.d.
6	82		0.398±0.0735	8.99±1.74^a
7	83		1.50±0.688^a	17.4±4.15
8	97		n.d.	n.d.
9	99		n.d.	n.d.
10	106		>100^a	>100^a
11	107		0.0274±0.0130	0.281±0.0083
12	108		0.0927±0.0186^a	0.220±0.0475

Data are **mean** ± SEM of three or two (^a) independent experiments. n.d.: not determined.

IT1t's affinity for CXCR4 was found superior to our analogues'. Triazole-bearing analogues **45**, **46** and **69** were found inactive against CXCR4 (entries 2–4). To our surprise, the more sterically hindered analogue **46** was more potent than its dimethyl congener **45** by about a log unit, suggesting that the presence of lipophilic bulk in this region might be beneficial to binding activity. This is corroborated by the work of Thoma and co-workers,⁵⁰ whereby cyclohexyl, cycloheptyl and cyclooctyl analogues proved more potent than the cyclopentyl analogue (IC_{50} = 48, 28, 28, 372 nM), while the 2-adamantyl analogue conserved some affinity (IC_{50} = 81 nM). Similarly, the absence of affinity observed for analogue **69** can be correlated with the low affinity obtained with Thoma's *n*-hexyl analogue (IC_{50} = 855 nM).

Both our piperidine analogues **83** and **82** showed limited antagonistic activities, with a slight preference for the fluoroethylated compound (entries 6–7). Together with the IC_{50} value of Thoma's 4-*tert*-butylcyclohexyl analogue (1533 nM), these data suggest that derivatisation of 6-membered rings at their 4-position is detrimental to some extent. However, assuming that the valuable pharmacokinetic properties of IT1t are transferred to its analogues, these could compensate analogue **82**'s suboptimal IC_{50} value (398 nM).

Finally, although it was found that substituting phenyl for cyclohexyl dramatically reduced the compound's binding to CXCR4,⁵⁰ we observe that addition of a fluorine atom *meta* or *para* on the aromatic ring offered satisfactory binding affinities and chemotaxis inhibitory powers (entries 11 and 12). Our assays displayed a slight preference for the *meta* analogue, further corroborating our hypotheses about the 4-position of 6-membered rings. Moreover, *ortho* analogue **106** exhibited no affinity for CXCR4 (IC_{50} > 100 μ M), which can be rationalised from our preliminary work (see section IV.1.2) (Figure 49).

In IT1t's binding mode, the binding compound **106** is hindered by the fluorine atom, as it might obstruct the important salt bridge between isothioureia's NH^+ and Asp97's carboxylate.

Nonetheless, the low IC_{50} value exhibited by analogue **107** (27.4 nM) is auspicious and warrant further investigation of the tracer-like qualities of this compound.

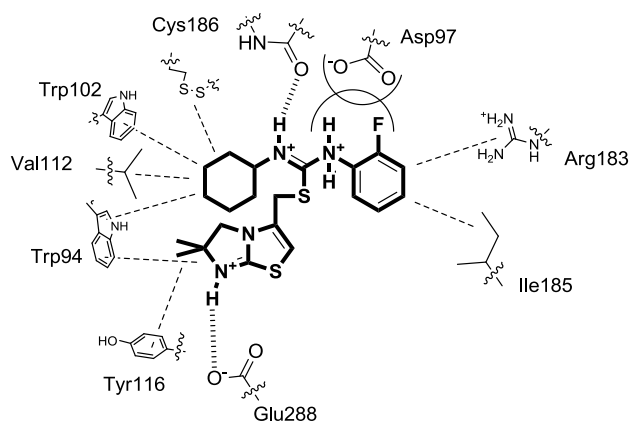


Figure 49. Schematic representation of plausible interactions between CXCR4 and *ortho* analogue **106**. Salt bridges and hydrogen bonds are represented as thick dash bonds, hydrophobic interactions are represented as thin dash bonds, steric hindrance is represented with curves.

5.2. Cellular uptake assays

Cellular uptake of gallium-68-chelated **110** was assessed in U87.CD4 cells transfected with CXCR4 (U87.CD4.CXCR4) (Figure 50).

60-min cellular uptake of the tracer showed no difference in uptake between CXCR4-expressing cells and isogenic CXCR4-low cells, which constitutes evidence that this compound has no affinity for CXCR4. Addition of 10 μM of IT1t showed a non-significant decrease of the cellular uptake, which further corroborates these negative results. This *in vitro* data support our hypothesis that IT1t analogue that present a long chain instead of a cyclohexyl are not well tolerated by CXCR4.

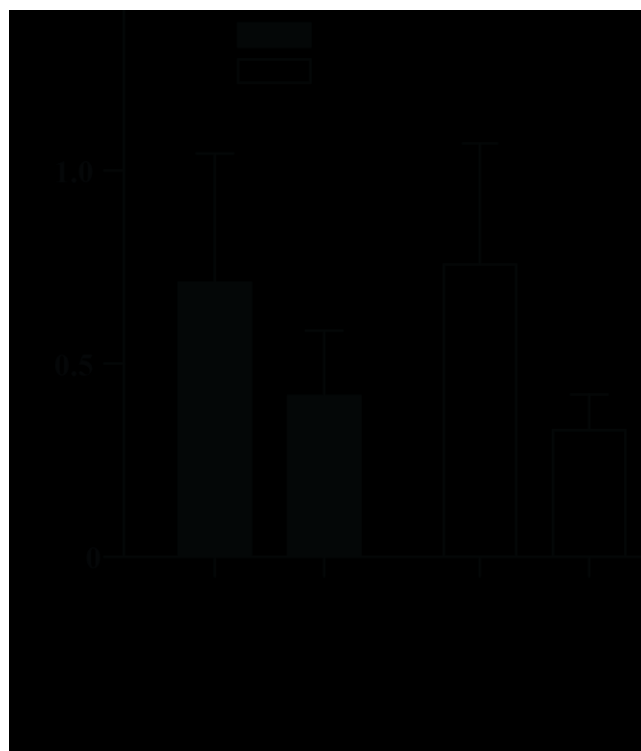


Figure 50. Gallium-68-chelated **110** *in vitro* uptake in U87.CD4 and U87.CD4.CXCR4 cells. Data are mean $\pm$ SD of three independent experiments and statistical significance was assessed using a 2-way ANOVA with Bonferroni post-tests ($P < 0.05$).

6. Conclusions

Using the recently disclosed structure of CXCR4 co-crystallised with IT1t, we were able to understand the nature of the interactions underlying the affinity of this compound for CXCR4. Because of the recognized flexibility of GPCRs, we chose to analyse the binding site by docking IT1t: this allowed us to identify one of IT1t's cyclohexyl as the moiety most likely to tolerate modifications. On this basis, we undertook efforts at synthesising a variety of IT1t analogues, precursors and reference compounds. Although some analogues proved challenging to produce, a small library of fluorinated compounds was prepared, amongst which analogues **107** and **108** exhibited high affinity for CXCR4 ($IC_{50} = 27.4$ and 92.7 nM, respectively). These compounds could in principle be radiolabelled by the novel nickel-mediated radiolabelling method developed by Ritter and co-workers.⁸⁸ Although less potent, fluoroethylpiperidine derivative **82** could be radiosynthesised from precursor **80** using the well-established fluoroalkylation procedure. Finally, we expect cyclobutyl analogues **97** and

99 to be potent, owing to the small size of their modification; these compounds could be radiosynthesised from the corresponding triflate or tosylate by substitution for [^{18}F]fluoride.

We successfully radiolabelled two tracers based on the structure of IT1t with fluorine-18 and gallium-68, and assessed the gallium-68-chelated **110** on a cell-based *in vitro* uptake assay, in which unfortunately no radioactivity uptake was observed.

CONCLUSIONS

In this research, we were interested in creating new PET probes specific for CXCR4 based on the structures of three known CXCR4 antagonists: tetradecapeptide TN14003, cyclopentapeptide FC131 and small molecule IT1t. We analysed their SARs based upon careful survey of the existing literature. This analysis was supplemented by *in silico* work, which allowed us to further our knowledge of the nature of the interactions between CXCR4 and its antagonists. Taken together, these data allowed the generation of twenty tracer candidates, five of which were selected for radiosynthesis and biologically evaluated to various extents (Figure 51).

Our TN14003-based tracer, [^{68}Ga]**22**, underwent comprehensive preclinical evaluation. Promising *in vitro* results were confirmed *in vivo* with a 5.3% ID/g uptake in CXCR4-expressing U87.CD4.CXCR4 tumours, which allowed for effective visualisation of the tumour by PET imaging. Compared to literature precedent, our tracer exhibited the highest tumour uptake. It can be argued that the tumour-to-muscle ratio of tracer [^{68}Ga]**22** is lower than that of Jacobson's tracers [^{64}Cu]**9** and [^{64}Cu]**10**,¹⁰⁵ nevertheless, a tumour-to-muscle ratio of ~ 10 , as the one exhibited by tracer [^{68}Ga]**22**, is deemed satisfactory for imaging purposes. Furthermore, accruing from their higher probability to decay by positron emission and shorter half-life, gallium-68-labelled probes result in lower doses to patients. However, kidney uptake (24% ID/g) is a challenge for clinical translation and needs to be precluded. To this end, the determination of the nature of the metabolites encountered during metabolite study (see section II.4.4) could provide guidance in the modification of the structure of TN14003. The low metabolism rate found with cyclopentapeptidic tracer [^{18}F]**35** implies that the residues indispensable for binding to CXCR4¹²⁹ are unlikely to need modification, which suggests that a tracer based on the structure of TN14003 and with an improved pharmacokinetic profile is achievable.

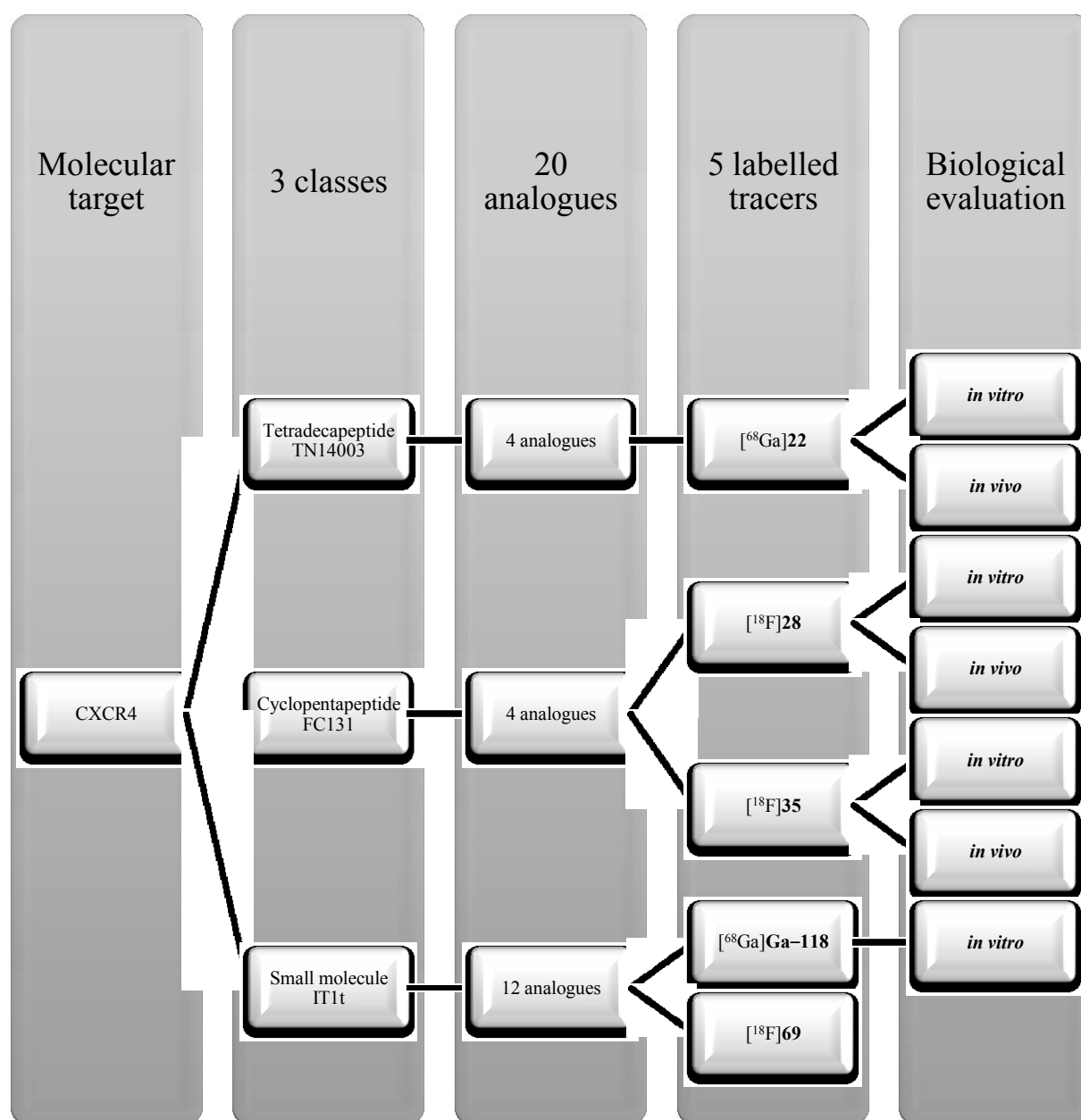


Figure 51. Summary of the work presented in this thesis.

The work we carried out on the cyclopentapeptidic class showed it was possible to produce fluorine-18-labelled FC131 analogues retaining affinity for CXCR4. In particular, both compounds [¹⁸F]28 and [¹⁸F]35 exhibited promising CXCR4-specific tumour uptake *in vitro*. The relatively low uptake in mouse organs and adequate *in vivo* stability were unfortunately counterbalanced by the lack of difference in tumour uptake between CXCR4-expressing and CXCR4-low cell lines, seemingly due to an excessive urinary excretion rate. Nevertheless, Wester and co-workers^{100,101} showed it was possible to obtain a radiotracer of satisfactory

pharmacokinetic profile with a cyclopentapeptidic targeting core. The reduced overall charge of the tracer they designed could be the key to reduced excretion. It would therefore be advantageous to substitute an arginine bioisoster for compound **35**'s Arg3. For instance, citrulline was used to replace arginine in analogues of tetradcapeptide TN14003,^{115,129} while De Marco and co-workers disclosed a sulfamoylated ornithine as arginine surrogate (Figure 52).¹⁷³ In addition, introduction of such additional unnatural amino acids in the chemical structure could further temper tracer metabolism.

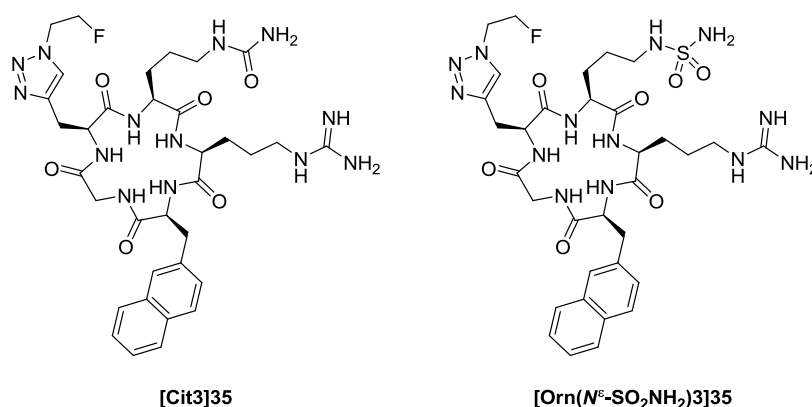


Figure 52. Proposed modifications to preclude detrimentally rapid renal excretion of cyclopentapeptidic tracer. Cit: citrulline. Orn: ornithine.

On top of this work on peptidic radiotracers for the imaging of CXCR4, we created a preliminary library of small molecule IT1t analogues for radiolabelling with fluorine-18 or gallium-68. In particular, analogues **107** and **108** exhibited IC₅₀ values derived from competitive binding assays less than 100 nM and could be radiosynthesised with Ritter's nickel-promoted radiofluorination.⁸⁸ The CXCR4 imaging community would undoubtedly benefit from further biological examination of a selection of these compounds, which have great pharmacokinetic potential.⁵⁰ Second-generation radiotracers based on IT1t could then be designed based on these biological data.

Overall, this project allowed us to explore the potential of three classes of compounds as PET tracers for CXCR4. Although only the gallium-68-labelled TN14003 tracer was successful in imaging tumours *in vivo*, our work offers interesting advances towards tracers based on the structures of FC131 and IT1t. While tracers [¹⁸F]**28** and [¹⁸F]**35** need to be improved to

Conclusions

reduce renal excretion, *in vivo* assessment of a selection of the IT1t-based molecules presented herein is eagerly expected.

It can be anticipated that exploration of other classes of CXCR4-binding compounds could provide tracers of interesting characteristics. As underlined previously (see section I.6), cyclams have attracted particular attention and resulted in two copper-64-labelled tracers, and fluorine-18- and gallium-68-labelled analogues are being developed in our laboratories as part of our on-going CXCR4 programme. Moreover, the plethora of small molecules derived from the original hit AMD3100³⁵ offers an interesting pool of molecules from which new tracers could be designed.

Peptides offer other sources of inspiration. Nanobodies exhibit pharmaceutical profiles more suitable to PET imaging than antibodies, while conserving high affinity for their target. For instance, anti-CXCR4 nanobodies 238D2 and 238D4 were radiolabelled with iodine-125.¹⁷⁴ However, due to the size of such species (~15 kDa), the selectivity of nanobody labelling is poor. Presumably, this could be circumvented by the use of histidine tag, a hexahistidine motif that could be added to one of the nanobody's termini. This technology was used for technetium-99m-labelling of antibody fragments,¹⁷⁵ and it could be anticipated that similar labelling method could be used with copper-64 or gallium-68. Therefore, a plasmid containing the sequence of the desired nanobody modified to exhibit the hexahistidine motif could be transfected to *Escherichia coli*, the resulting expressed peptide purified and used for radiolabelling.

Another unexplored strategy for the production of CXCR4-targeted radiotracers is the exploitation of HIV-1's V3 loop (see section I.2.3). This 25-amino acid peptide could theoretically be synthesised with minimum effort in a chemistry laboratory and derivatised at one of its termini with a chelating motif such as NO2A or DO3A. The resulting precursor could then be radiolabelled with various metals (*e.g.*, gallium-68, aluminium fluoride-18, copper-64). Would it not be a delectable revenge on HIV to transform one of its most lethal weapons into a medical tool?

EXPERIMENTAL PROCEDURES

1. General

All reagents and solvents were purchased from commercial sources, namely: Sigma-Aldrich, Fluka, Activate Scientific, TCI Europe, Apollo Scientific, Fischer, Cambridge Bioscience or Fluorochem and used without further purification. Precursor **21** was obtained from Peptide Protein Research Ltd (Fareham, UK). Conventional heating was carried out with a heating block or an oil bath and the temperature was measured using an alcohol or a digital thermometer. A Mettler Toledo pH meter was used for the preparation of buffers.

1.1. Cold chemistry

Compounds FEA (**27**),¹⁷⁶ *n*-C₈F₁₇CH₂CH₂N₃ (**135**),¹⁷⁷ BnN₃ (**136**),¹⁴⁸ 2-fluoroethyl tosylate (**81**),¹⁷⁸ 5-fluoropent-1-yne (**40**),⁷⁰ *tert*-butyl 2-{4,7-bis[2-(*tert*-butoxy)-2-oxoethyl]-1,4,7,10-tetraazacyclododecan-1-yl}acetate (**121**),¹⁶⁶ DOTA(*O*-Et)(*O*-*t*Bu)₃ (**123**)¹⁶⁷ and DOTA(*t*Bu)₃ (**113**)¹⁶⁷ were prepared following literature procedures. Peptides were shaken on an IKA KS 130-basic device. **Flash column chromatography** was carried out on silica gel (Fluka 230-400 mesh, for flash chromatography). **Thin layer chromatography** was performed on aluminum plates pre-coated with silica (200 μm, 60 F₂₅₄) which were visualised by quenching of ultraviolet fluorescence ($\lambda_{\text{max}} = 254 \text{ nm}$), by charring with various dips (KMnO₄, ninhydrin, paranisaldehyde or bromocresol green) or with iodine. **Melting points** were determined on a Khofler hot stage and are uncorrected. **Specific rotations** ($[\alpha]_D^{25}$) were recorded on a Perkin-Elmer 241 polarimeter at 589 nm (Na D-line) with a path length of 0.5 dm. Concentrations (*c*) are quoted in g/100 mL and specific rotations are quoted in units of 10⁻¹ °.cm².g⁻¹ at 20 °C. **Infrared spectra** were recorded neat on Perkin-Elmer Paragon 1000 Fourier transform spectrometer with automated background subtraction. Only selected absorptions are reported. ¹H, ¹³C, ¹⁹F and ⁷¹Ga NMR spectra were obtained at 300 K on Bruker AV-400, DRX-400 or AV-500 instruments. Chemical shifts (δ) are given in parts per million (ppm) as referenced to the appropriate residual solvent peak (for Me₂SO-*d*₆ ¹H $\delta = 2.50 \text{ ppm}$ and ¹³C $\delta = 39.5 \text{ ppm}$, for MeOH-*d*₄ ¹H $\delta = 3.31 \text{ ppm}$ and ¹³C $\delta = 49.0 \text{ ppm}$, for CDCl₃ ¹H $\delta = 7.26 \text{ ppm}$ and ¹³C $\delta = 77.2 \text{ ppm}$),¹⁷⁹ to CFC₃ (¹⁹F $\delta = 0 \text{ ppm}$) or to Ga(NO₃)₃ (⁷¹Ga $\delta = 0 \text{ ppm}$). ¹³C chemical shifts are assigned as s, d, t and q for C, CH, CH₂ and CH₃,

Experimental procedures

respectively. Coupling constant (J) are given in Hertz (Hz). **Mass spectra** were obtained in electrospray ionisation mode on a Micromass LCT Premier and in electron ionisation and chemical ionisation modes on a Micromass AutoSpec Premier. HRMS values are valid up to ± 5 ppm. **Preparative UV-HPLC** was carried out using a Waters 600 pump and Waters Empower Pro software equipped with a linear Water 2487 UV detector ($\lambda = 254$ and 270 nm or $\lambda = 254$ and 210 nm) and a Phenomenex Luna C18(2) $5\ \mu$ 75×30.00 mm HPLC column. Injection loop $5000\ \mu\text{L}$. Mobile phase (A) $\text{H}_2\text{O} + 0.1\%$ TFA and (B) $\text{MeCN} + 0.1\%$ TFA. Flow rate $20\ \text{mL}\cdot\text{min}^{-1}$. **Semi-preparative UV-HPLC** was carried out using the same system as for preparative UV-HPLC with a Phenomenex Luna C18(2) $5\ \mu$ 100×10.00 mm HPLC column. Method: gradient 5% to 95% B over 20 min. Flow rate $3.15\ \text{mL}\cdot\text{min}^{-1}$. **Analytical UV-HPLC** was carried out using a Hewlett Packard Series 1100 G1312A pump and Lablogic Laura 3 software equipped with a linear Hewlett Packard Series 1100 G1314A UV detector ($\lambda = 254$ nm or $\lambda = 210$ nm) and a Phenomenex Luna C18(2) $5\ \mu$ 150×4.6 mm HPLC column. Injection loop $20\ \mu\text{L}$. Mobile phase (A) $\text{H}_2\text{O} + 0.1\%$ TFA and (B) $\text{MeCN} + 0.1\%$ TFA. Gradient 5 – 95% B over 10 min. Flow rate $1\ \text{mL}\cdot\text{min}^{-1}$.

1.2. Radiochemistry

^{18}F Fluoride was acquired from PETNet Solutions (Manchester, UK) or produced using 11 MeV Siemens Eclipse HP cyclotrons (^{18}O enriched water, $>98\%$ ^{18}O atom, was purchased from Rotem). ^{68}Ga GaCl_3 was eluted with a $0.1\ \text{M}$ HCl (Merck Ultrapur) solution from a Eckert&Ziegler Isotope Products IGG100-10M Gallium-68 Generator (nuclide crystal source: germanium-68 adsorbed to TiO_2 bed). **Semi-preparative UV-radio-HPLC** purifications were run using a Phenomenex Luna C18(2) $5\ \mu$ 100×10.00 mm HPLC column. Mobile phase (A) $\text{H}_2\text{O} + 0.1\%$ TFA and (B) $\text{MeCN} + 0.1\%$ TFA. Flow rate $3.15\ \text{mL}\cdot\text{min}^{-1}$. **Analytical UV-radio-HPLC** analyses were run using a mobile phase (A) $\text{H}_2\text{O} + 0.1\%$ TFA and (B) $\text{MeCN} + 0.1\%$ TFA. Injection loop $20\ \mu\text{L}$. Flow rate $1\ \text{mL}\cdot\text{min}^{-1}$.

1.3. Biology

Cell culture: the human cell line Jurkat clone E6-1 was purchased from ATCC (USA) and maintained in RPMI media (Invitrogen, USA). U87 glioblastoma cells stably transfected with CD4 (U87.CD4) or both CD4 and CXCR4 (U87.CD4.CXCR4) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr Erman Mandaci. This experimental model is commonly used for the evaluation of drug/probe targeting CXCR4. U87.CD4 and U87.CD4.CXCR4 cells were maintained in low

glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA). The medium for U87.CD4 was supplemented with 300 $\mu\text{g/mL}$ G418 (Sigma, USA), whilst U87.CD4.CXCR4 was cultured in media containing 300 $\mu\text{g/mL}$ G418 and 1 $\mu\text{g/mL}$ puromycin (Sigma, USA). All cell lines were cultured in the presence of 10% foetal bovine serum (FBS) (Sigma-Aldrich, USA), 2 mM L-glutamine (Invitrogen, USA) and 100 U/mL penicillin-streptomycin (Invitrogen, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. **Statistical analysis:** results were expressed as mean and standard error from the mean (SEM). Two-tailed unpaired student's t tests were used to quantify statistical significance using GraphPad Prism 5, where $P < 0.05$.

2. Computational chemistry

Computational work was carried out as described in the text (see sections III.1.3, IV.1.2 and IV.3.5.1).

3. Cold chemistry

3.1. Peptidic molecules

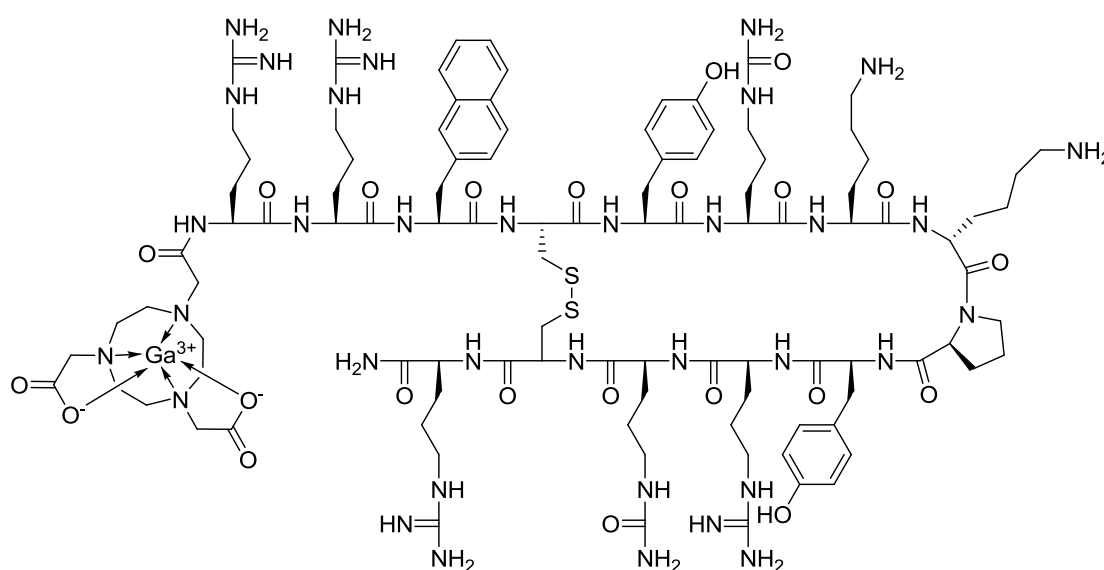
3.1.1. Compound **22**

Name: Ga^{III}–NOT2A – Arg – Arg – Nal – Cys – Tyr – Cit – Lys – D-Lys – Pro – Tyr – Arg – Cit – Cys – Arg – NH₂ 4.4-4.12-disulfide .xTFA

Chemical formula: C₁₀₂H₁₅₈GaN₃₆O₂₃S₂⁺

Exact mass: 2388.0992

Molecular weight of M.7TFA: 3187.58



Following a procedure reported in the literature.¹²⁴ To a solution of **21** (3.09 mg, 0.92 μmol, 1.0 equiv.) in water (276 μL) was added a solution of GaCl₃ (0.486 mg, 2.76 μmol, 3.0 equiv.) in sodium acetate buffer (92 μL, 0.1 M, pH 4.0). The reaction mixture allowed to stir at 80 °C for 10 min before being purified by semi-preparative HPLC, gradient 5–95% B over 20 min, to afford 1.74 mg (0.566 μmol, 62%) of the title compound as a white amorphous powder; δ_F (471 MHz, D₂O) –75.8 (TFA); δ_{Ga} (122 MHz, D₂O) –178.3, –177.9 (two isomers); *m/z* (ESI) (rel. int.) 798 (MH₂³⁺, 20), 598 (MH₃⁴⁺, 100), 479 (MH₄⁵⁺, 45), 342 (MH₅⁶⁺, 40) [Found MH₃⁴⁺, 598.0311. C₁₀₂H₁₅₈GaN₃₆O₂₃S₂⁺ requires MH₃⁴⁺, 598.0323; Δ = – 2.0 ppm]

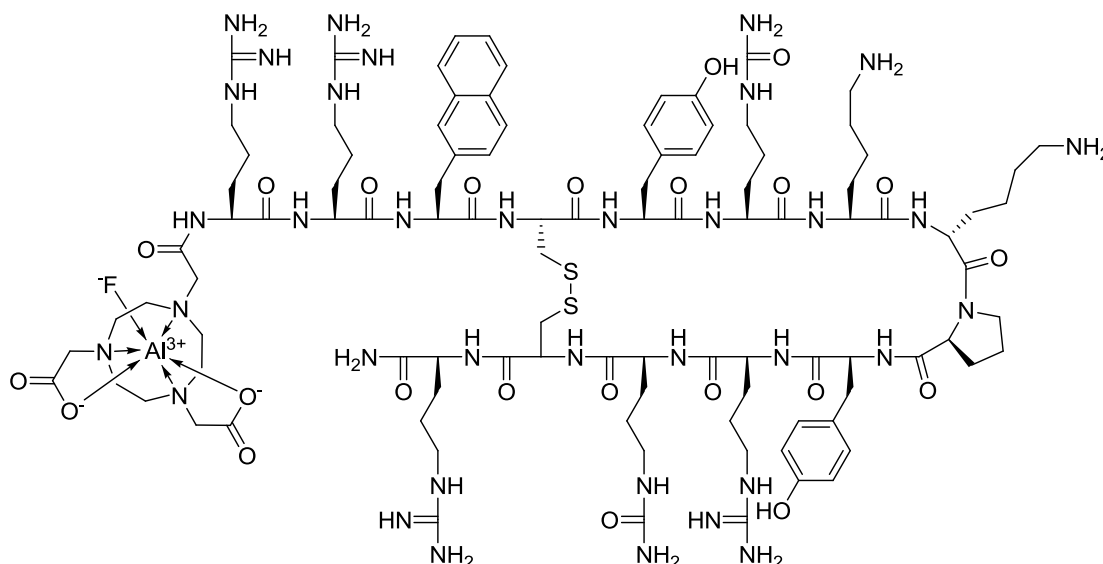
3.1.2. Compound **23**

Al^{III}F–NOT2A – Arg – Arg – Nal – Cys – Tyr – Cit – Lys – D-Lys – Pro – Tyr – Arg – Cit – Cys – Arg – NH₂ 4.4-4.12-disulfide .xTFA

Chemical formula: C₁₀₂H₁₅₈AlFN₃₆O₂₃S₂

Exact mass: 2365.1541

Molecular weight of M.6TFA: 3050.82



Following a procedure reported in the literature.¹²⁴ To a solution of AlCl₃ (0.388 mg, 2.91 μmol, 3.0 equiv.) in sodium acetate buffer (1.455 mL, 0.1 M, pH 4.0) was added a solution of NaF (0.135 mg, 3.20 μmol, 3.3 equiv.) in sodium acetate buffer (1.067 mL, 0.1 M, pH 4.0) and the reaction mixture was allowed to stir at 100 °C for 15 min. A solution of **21** (3.25 mg, 0.97 μmol, 1.0 equiv.) in sodium acetate buffer (388 μL, 0.1 M, pH 4.0) was then added before allowing the reaction to stir at 100 °C for 15 min. The reaction mixture was purified by semi-preparative HPLC, gradient 5–95% B over 20 min, to afford 1.59 mg (0.521 μmol, 54%) of the title compound as a white amorphous powder; δ_F (471 MHz, D₂O) –75.8 (CF₃CO₂H), –122.5 (AlF); *m/z* (ESI) (rel. int.) 790 (MH₃³⁺, 35), 593 (MH₄⁴⁺, 100), 474 (MH₅⁵⁺, 95).

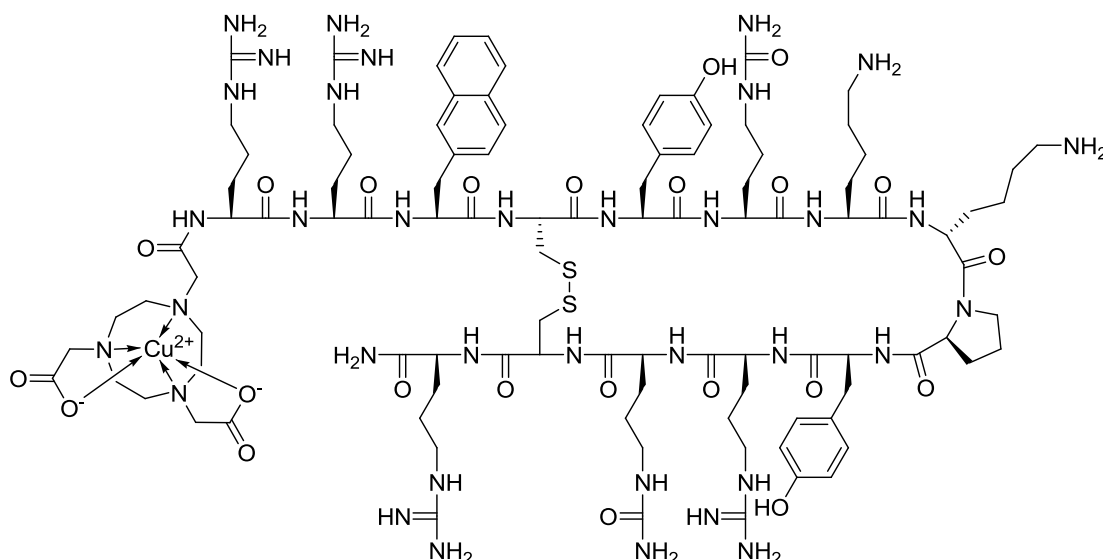
3.1.4. Compound 25

Name: Cu^{II}-NOT2A – Arg – Arg – Nal – Cys – Tyr – Cit – Lys – D-Lys – Pro – Tyr – Arg – Cit – Cys – Arg – NH₂ 4.4-4.12-disulfide .xTFA

Chemical formula: C₁₀₂H₁₅₈CuN₃₆O₂₃S₂

Exact mass: 2382.1038

Molecular weight of M.6TFA: 3068.39



Following the procedure for **24**, **21** (3.09 mg, 0.92 μ mol, 1.0 equiv.) in ammonium acetate buffer (200 μ L, 65 mM, pH 6.0) and CuCl₂·2H₂O (1.57 mg, 9.2 μ mol, 10.0 equiv.) in ammonium acetate buffer (200 μ L, 65 mM, pH 6.0) afforded 0.87 mg (0.32 μ mol, quantitative) of the title compound as a light blue amorphous powder after purification by semi-preparative HPLC, gradient 5–95% B over 20 min; δ_F (471 MHz, D₂O) –75.74; *m/z* (ESI) (rel. int.) 1193 (MH₂²⁺, 5), 796 (MH₃³⁺, 40), 597 (MH₄⁴⁺, 100), 478 (MH₅⁵⁺, 80).

3.1.5. General procedure for the synthesis of cyclopentapeptides

In a syringe equipped with a plastic filter and a stopper at its bottom, H-Gly-2CITrt resin (0.46 mmol/g, 100 μ mol) was swollen in DMF for 30 min in a shaker and the solvent removed by filtration. HOBt (40 mg), HBTU (113 mg), Fmoc-protected amino acid (300 μ mol) and DIPEA (52 μ L) were mixed in 3.2 mL DMF, this coupling mixture was added to the syringe and the reaction mixture was shaken at r.t. for 2 h. The resin was then washed with DMF (3×30 s), the Fmoc protecting group was removed by treatment of the resin with 20% piperidine in DMF (4 mL) for 30 min and the resin was washed again with DMF (3×30 s). The sequence coupling – washing – deprotection – washing was repeated with the appropriate Fmoc-protected amino acids until the desired resin-bound pentapeptide was

Experimental procedures

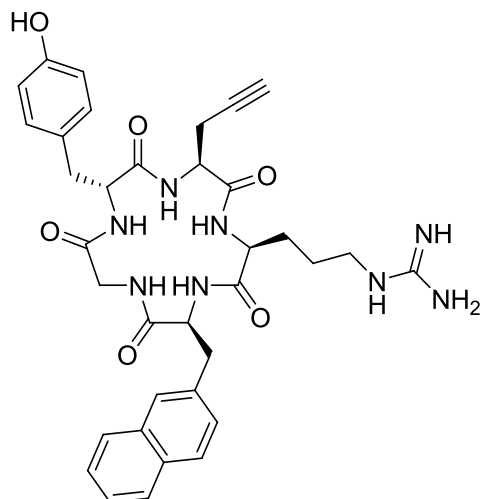
synthesized. The pentapeptide was cleaved from the resin by treatment with a 1:1:3 mixture of AcOH–TFE–CH₂Cl₂ (1×2 h and 3×2 min, 10 mL each) and the combined filtrates were concentrated under reduced pressure. To a solution of the resulting unprotected linear pentapeptide and NaHCO₃ (60 mg) in DMF (40 mL) at –40 °C was added DPPA (100 µL) and the reaction mixture was allowed to stir at r.t. for 4 days after which DMF was removed under reduced pressure. The residue was then suspended in MeOH–CHCl₃ (9:1) (5 mL) and filtered through a small column of basic alumina. The filtrate was concentrated *in vacuo* and the residue was taken up in a minimum of CH₂Cl₂ and precipitated with Et₂O. The solid was filtered and washed with Et₂O, affording the protected cyclopentapeptide in good to high yields (73 to >99%) as a white amorphous powder. The peptide was dissolved in TFA–TIS–H₂O (190:5:5) (10 mL), the reaction mixture was allowed to stir at r.t. for 6 h and concentrated *in vacuo* and the residue was suspended in Et₂O before filtering to afford a white amorphous powder. The crude product was purified by preparative HPLC, gradient 5–95% B over 10 min. The collected product fraction was freeze-dried to afford the TFA salt of the desired product as a white amorphous powder in moderate to good yield (12–45%). Alkyne-containing deprotected cyclopentapeptides (6.4 µmol) underwent Huisgen alkyne-azide cyclization with CuSO₄·5H₂O (0.32 mg, 1.3 µmol, 0.2 equiv.), sodium ascorbate (5.6 mg, 32 µmol, 5 equiv.), FEA (**27**) (250 µL, 1.84 M in DMF) and sodium acetate buffer (250 µL, pH 5, 250 mM) at r.t. over a week. The reaction mixture was subjected to the same HPLC purification conditions as described above and freeze-dried to afford the triazole "clicked" product as a white amorphous powder in good to quantitative yield (89 – >99%).

3.1.6. Compound 26

Name: *cyclo*(– Gly – D-Tyr – Prg – Arg – Nal –) .xTFAChemical formula: C₃₅H₄₀N₈O₆

Exact mass: 668.3071

Molecular weight of M.TFA: 782.77



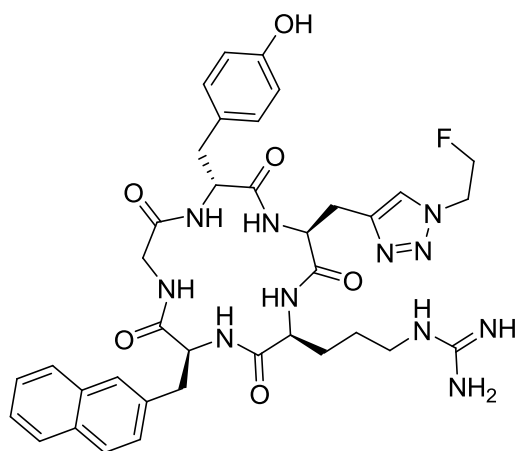
m/z (ESI) (rel. int.) 671 (10), 670 (40), 669 (MH⁺, 100) [Found MH⁺, 669.3127. C₃₅H₄₀N₈O₆ requires MH⁺, 669.3149; Δ = –3.3 ppm].

3.1.7. Compound 28

Name: *cyclo*(– Gly – d-Tyr – Fta – Arg – Nal –) .xTFAChemical formula: C₃₇H₄₄FN₁₁O₆

Exact mass: 757.3460

Molecular weight of M.TFA: 871.84



δ_H (500 MHz, CDCl₃–MeOH-*d*₄ 1:1) 7.87 – 6.59 (12H, m, aromatics), 4.91 – 1.32 (22H, m, non-aromatics); δ_F (376 MHz, MeOH-*d*₄–D₂O 1:1) –76.2 (s, TFA), –222.6 (tt, *J* = 45.8 and

Experimental procedures

25.6 Hz, $-\text{CH}_2\text{F}$); m/z (ESI) (rel. int.) 820 (MCu^+ , 60), 758 (MH^+ , 100) [Found MH^+ , 758.3530. $\text{C}_{37}\text{H}_{44}\text{FN}_{11}\text{O}_6$ requires MH^+ , 758.3538; $\Delta = -1.1$ ppm].

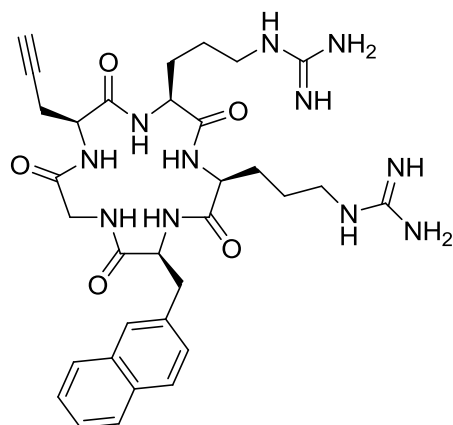
3.1.8. Compound 34

Name: *cyclo*(– Gly – Prg – Arg – Arg – Nal –) .xTFA

Chemical formula: $\text{C}_{32}\text{H}_{43}\text{N}_{11}\text{O}_5$

Exact mass: 661.3449

Molecular weight of M.2TFA: 889.80



m/z (ESI) (rel. int.) 662 (MH^+ , 20), 332 (MH_2^{2+} , 100) [Found MH^+ , 662.3509. $\text{C}_{32}\text{H}_{43}\text{N}_{11}\text{O}_5$ requires MH^+ , 662.3527; $\Delta = -2.7$ ppm].

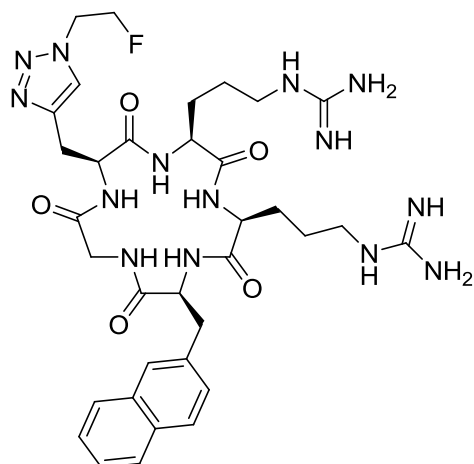
3.1.9. Compound 35

Name: *cyclo*(– Gly – Fta – Arg – Arg – Nal –) .xTFA

Chemical formula: $\text{C}_{34}\text{H}_{47}\text{FN}_{14}\text{O}_5$

Exact mass: 750.3838

Molecular weight of M.2TFA: 978.87



δ_F (376 MHz, MeOH- d_4 -D₂O 1:1) -76.2 (s, TFA), -223.2 (tt, $J = 48.5$ and 28.4 Hz, $-\text{CH}_2\text{F}$); m/z (ESI) (rel. int.) 751 (MH^+ , 10), 593 (100) [Found MH^+ , 751.3904 . $\text{C}_{34}\text{H}_{47}\text{FN}_{14}\text{O}_5$ requires MH^+ , 751.3916 ; $\Delta = -1.6$ ppm].

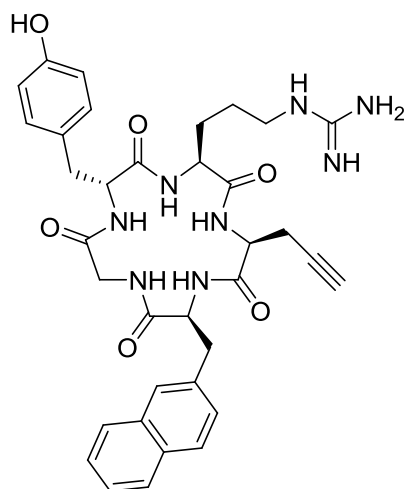
3.1.10. Compound 36

Name: *cyclo*(– Gly – D-Tyr – Arg – Prg – Nal –) .xTFA

Chemical formula: $\text{C}_{35}\text{H}_{40}\text{N}_8\text{O}_6$

Exact mass: 668.3071

Molecular weight of M.TFA: 782.77



m/z (ESI) (rel. int.) 671 (10), 670 (40), 669 (MH^+ , 100) [Found MH^+ , 669.3149 . $\text{C}_{35}\text{H}_{40}\text{N}_8\text{O}_6$ requires MH^+ , 669.3149 ; $\Delta = 0.4$ ppm].

Experimental procedures

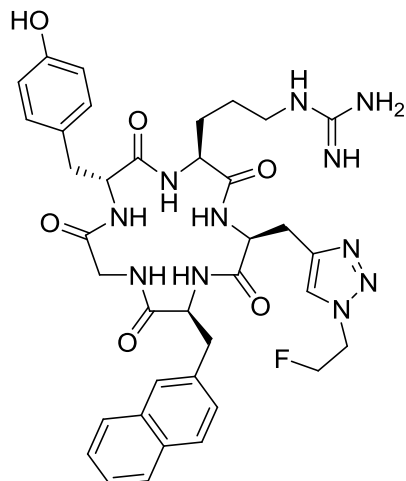
3.1.11. Compound 37

Name: *cyclo*(– Gly – D-Tyr – Arg – Fta – Nal –) .xTFA

Chemical formula: $C_{37}H_{44}FN_{11}O_6$

Exact mass: 757.3460

Molecular weight of M.TFA: 871.84



δ_F (376 MHz, MeOH- d_4 -D $_2$ O 1:1) –76.2 (s, TFA), –223.2 (tt, J = 46.8 and 27.3 Hz, –CH $_2$ F); m/z (ESI) (rel. int.) 758 (MH $^+$, 100), 759 (50) [Found MH $^+$, 758.3568. $C_{37}H_{44}FN_{11}O_6$ requires MH $^+$, 758.3538; Δ = 4.0 ppm].

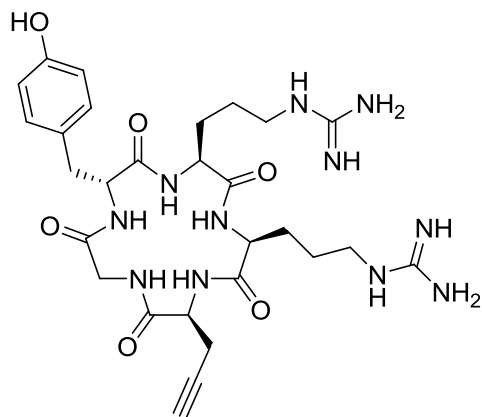
3.1.12. Compound 38

Name: *cyclo*(– Gly – D-Tyr – Arg – Arg – Prg –) .xTFA

Chemical formula: $C_{28}H_{41}N_{11}O_6$

Exact mass: 627.3241

Molecular weight of M.2TFA: 855.74



m/z (ESI) (rel. int.) 628 (MH $^+$, 50), 315 (MH $_2^{2+}$, 100) [Found MH $^+$, 628.3312. $C_{28}H_{41}N_{11}O_6$ requires MH $^+$, 628.3320; Δ = –1.3 ppm].

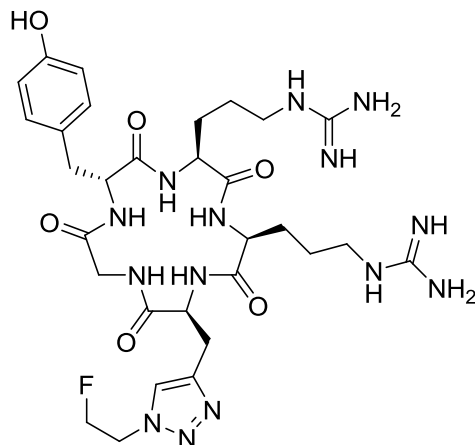
3.1.13. Compound 39

Name: *cyclo*(– Gly – D-Tyr – Arg – Arg – Fta –) .xTFA

Chemical formula: C₃₀H₄₅FN₁₄O₆

Exact mass: 716.3631

Molecular weight of M.2TFA: 944.81



δ_F (471 MHz, MeOH-*d*₄-D₂O 1:1) –76.4 (s, TFA), –223.9 (tt, *J* = 48.6, 27.1 Hz, –CH₂F); *m/z* (ESI) (rel. int.) 718 (15), 717 (MH⁺, 40) [Found MH⁺, 717.3704. C₃₀H₄₅FN₁₄O₆ requires MH⁺, 717.3719; Δ = 1.4 ppm].

3.2. Non-peptidic molecules

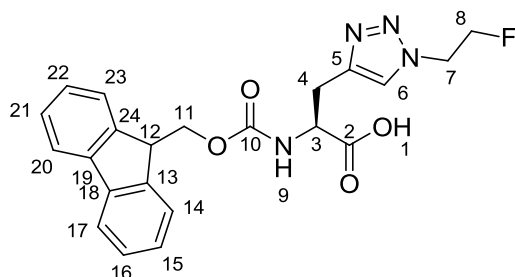
3.2.1. Compound 32

Name: (2*S*)-2-{[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino}-3-[1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-yl]propanoic acid

Chemical formula: C₂₂H₂₁FN₄O₄

Exact mass: 424.1547

Molecular weight: 424.4249



The compound was synthesised following a modified literature procedure.¹⁸⁰ To a solution of commercially available (*S*)-2-{[(9*H*-fluoren-9-yl)methoxy]carbonylamino}pent-4-ynoic acid **33** (5.08 g, 15.2 mmol, 1.0 equiv.) and FEA (**27**) (91.2 mL at 0.5 M in DMF, 45.6 mmol,

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3.0 equiv.) were added CuI (5.78 g, 30.4 mmol, 2.0 equiv.) and DIPEA (10.4 mL, 60.8 mmol, 4.0 equiv.) and the reaction mixture was allowed to stir at r.t. under an atmosphere of N₂ for 2 h. The reaction mixture was diluted with 500 mL of 0.5 N HCl_(aq) and extracted with ethyl acetate (100 mL). The organic layer was washed with 0.5 N HCl_(aq) (5×500 mL), dried over MgSO₄ and concentrated *in vacuo* to afford 6.01 g (14.2 mmol, 93%) of the title compound as an amorphous brown powder; **R_f** 0.33 (10:90:1 methanol – methylene chloride – acetic acid); [α]_D²⁰ –5.0 (*c* 0.08, MeOH); $\nu_{\text{max}}/\text{cm}^{-1}$ 1713 (s, C=O), 1522 (m, N–H), 1450 (s), 1221 (s, C–F), 1044 (s), 739 (vs); δ_{H} (400 MHz, MeOD) 7.80 (1H, s, H-6), 7.79 (2H, d, *J* = 7.4 Hz, H-17 and H-20), 7.63 (2H, m, H-14 and H-23), 7.39 (2H, dd, *J* = 7.4, 7.4 Hz, H-16 and H-21), 7.31 (2H, dd, *J* = 7.4, 7.4 Hz, H-15 and H-22), 4.76 (2H, dt, *J* = 47.3, 4.6 Hz, H-8), 4.67 (2H, dt, *J* = 27.1, 4.3 Hz, H-7), 4.54 (1H, dd, *J* = 9.1, 4.8 Hz, H-3), 4.30 (2H, d, *J* = 7.1 Hz, H-11), 4.20 (1H, t, *J* = 7.0 Hz, H-12), 3.40 – 3.10 (2H, m, H-4); δ_{C} (101 MHz, MeOD) 173.1 (s, C-2), 157.0 (s, C-10), 143.9 (s, C-13 and C-24), 143.6 (s, C-5), 141.2 (s, C-18 and C-19), 127.4 (d, C-16 and C-21), 126.8 (d, C-15 and C-22), 124.9 (d, C-14 and C-23), 123.6 (d, C-6), 119.5 (d, C-17 and C-20), 81.4 [d (d, *J* = 170.6 Hz), C-8], 66.7 (t, C-11), 53.7 (d, C-3), 50.4 [d (d, *J* = 20.3 Hz), C-7], 46.9 (d, C-8), 27.4 (t, C-4). δ_{F} (471 MHz, MeOD) –223.7 (tt, *J* = 47.1, 27.2 Hz); **m/z** (ESI) (rel. int.) 426 (25), 425 (MH⁺, 100) [Found MH⁺, 425.1616. C₂₂H₂₁FN₄O₄ requires MH⁺, 425.1625; Δ = –2.1 ppm].

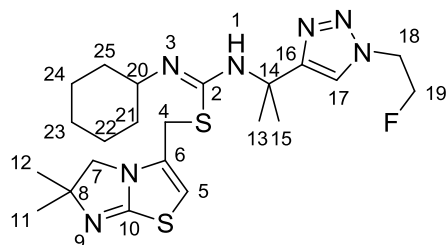
3.2.2. Compound 45

Name: *N'*-Cyclohexyl-*N*-(2-[1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-yl]propan-2-yl)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl]sulfanyl]methanimidamide .xTFA

Chemical formula: C₂₂H₃₄FN₇S₂

Exact mass: 479.2301

Molecular weight of M.3TFA: 821.75



Following the procedure for **106**, chloride **52** (28.0 mg, 0.12 mmol, 1.0 equiv.), thiourea **66** (36.7 mg, 0.12 mmol, 1.0 equiv.) and anhydrous MeCN (1.32 mL) afforded 42.9 mg (0.052 mmol, 43%) of the title compound as a yellow oil after purification by preparative HPLC, gradient 20–40% B over 10 min; $\nu_{\text{max}}/\text{cm}^{-1}$ 2938 (w, C–H), 2864 (w, Cy C–H), 1667

(s, C=N), 1125 (s, C-F); δ_{H} (400 MHz, MeOH- d_4) 8.20 (1H, s, H-17), 6.97 (1H, s, H-5), 4.90 – 4.71 (4H, m, H-18 and H-19), 4.60 (2H, s, H-4), 4.33 (2H, s, H-7), 3.92 (1H, br s, H-20), 1.86 (6H, s, H-13 and H-15), 1.85 – 1.62 (5H, m, H-21, H-22, H-23, H-24 and H-25), 1.62 (6H, s, H-11 and H-12), 1.60 – 1.34 (5H, m, H-21, H-22, H-23, H-24 and H-25); δ_{C} (101 MHz, MeOH- d_4) 169.2 (s, C-2), 163.6 (s, C-10), 160.9 [s (q, $J = 35.9$ Hz), $\text{CF}_3\text{CO}_2\text{H}$], 149.7 (s, C-16), 131.8 (s, C-6), 123.0 (d, C-17), 116.5 [s (q, $J = 293.4, 292.8$ Hz), $\text{CF}_3\text{CO}_2\text{H}$], 110.3 (d, C-5), 81.3 [t (d, $J = 170.9$ Hz), C-19], 69.9 (s, C-8), 58.3 (t, C-7), 56.8 (d, C-20), 55.2 (s, C-14), 50.8 [t (d, $J = 20.0$ Hz), C-18], 32.1 (t, C-21 and C-25), 28.9 (t, C-4), 26.7 (q, C-11 and C-12), 26.1 (q, C-13 and C-15), 24.4 (t, C-23), 23.7 (t, C-22 and C-24); δ_{F} (376 MHz, MeOH- d_4) –76.96 (TFA), –224.7 (tt, $J = 46.8, 27.2$ Hz); m/z (ESI) (rel. int.) 483 (5), 482 (15), 481 (30), 480 (MH^+ , 100), 325 (30) [Found MH^+ , 480.2374. $\text{C}_{22}\text{H}_{34}\text{FN}_7\text{S}_2$ requires MH^+ , 480.2379; $\Delta = -1.0$ ppm].

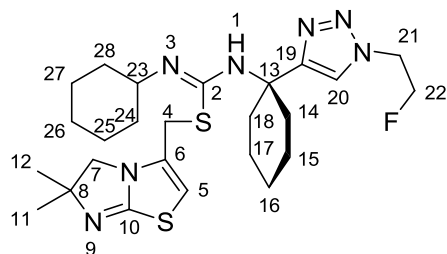
3.2.3. Compound 46

Name: *N'*-Cyclohexyl-*N*-{1-[1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-yl]cyclohexyl}[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl]sulfanyl]methanimidamide .xTFA

Chemical formula: $\text{C}_{25}\text{H}_{38}\text{FN}_7\text{S}_2$

Exact mass: 519.2614

Molecular weight of M.3TFA: 861.81



Following the procedure for **106**, chloride **52** (79.8 mg, 0.33 mmol, 1.0 equiv.), thiourea **67** (116 mg, 0.33 mmol, 1.0 equiv.) and anhydrous MeCN (3.3 mL) afforded 89.8 mg (0.104 mmol, 32%) of the title compound as a yellow oil after purification by preparative HPLC, gradient 20–40% B over 10 min; $\nu_{\text{max}}/\text{cm}^{-1}$ 3130 (w, ar C–H), 2937 (w, C–H), 1669 (m, C=N), 1127 (C–F); δ_{H} (400 MHz, MeOH- d_4) 8.27 (1H, s, H-20), 6.96 (1H, s, H-5), 4.93 – 4.74 (4H, m, H-21 and H-22), 4.53 (2H, s, H-4), 4.31 (2H, s, H-7), 3.93 (1H, br s, H-23), 2.58 – 2.41 (2H, m, H-14 and H-18), 2.27 – 2.12 (2H, m, H-14 and H-18), 1.91 – 1.64 (6H, m, H-15, H-17, H-24, H-25, H-27, H-28), 1.60 (6H, s, H-11 and H-12), 1.58 – 1.30 (10H, m, H-15, H-16, H-17, H-24, H-25, H-26, H-27, H-28); δ_{C} (101 MHz, MeOH- d_4) 169.2 (s, C-2), 163.3 (s, C-10), 160.8 [s (q, $J = 35.4$ Hz), $\text{CF}_3\text{CO}_2\text{H}$], 148.2 (s, C-19), 131.6 (s, C-6), 124.2 (d, C-

Experimental procedures

20), 116.5 [s (q, $J = 291.7$ Hz), $\text{CF}_3\text{CO}_2\text{H}$], 110.3 (d, C-5), 81.3 [t (d, $J = 170.9$ Hz), C-22], 69.9 (s, C-8), 58.3 (s, C-13), 58.2 (t, C-7), 56.5 (d, C-23), 50.9 [t (d, $J = 20.0$ Hz), C-21], 33.9 (t, C-14 and C-18), 32.2 (t, C-24 and C-28), 28.8 (t, C-4), 26.7 (q, C-11 and C-12), 24.5 (t, C-15 and C-17), 23.6 (t, C-16 and C-26), 21.6 (t, C-25 and C-27); δ_{F} (376 MHz, $\text{MeOH}-d_4$) – 76.9 (s, TFA), –224.8 (tt, $J = 46.3, 27.4$ Hz); m/z (ESI) (rel. int.) 522 (5), 521 (10), 520 (MH^+ , 25), 224 (60), 208 (100) [Found MH^+ , 520.2687. $\text{C}_{25}\text{H}_{38}\text{FN}_7\text{S}_2$ requires MH^+ , 520.2692; $\Delta = -1.0$ ppm].

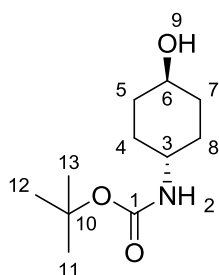
3.2.4. Compound **47**^{175,176}

Name: *tert*-Butyl *N*-(trans-4-hydroxycyclohexyl)carbamate^{181,182}

Chemical formula: $\text{C}_{11}\text{H}_{21}\text{NO}_3$

Exact mass: 215.1521

Molecular weight: 215.29

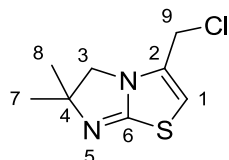


To a biphasic mixture of commercially available ammonium chloride salt **56** (1.0 g, 6.6 mmol, 1.0 equiv.) and Na_2CO_3 (1.82 g, 13.2 mmol, 2 equiv.) in water (20 mL) and CH_2Cl_2 (20 mL) under vigorous stirring was added Boc_2O (1.44 g, 6.6 mmol, 1.0 equiv.) portionwise and the reaction mixture was allowed to stir overnight. The organic layer was then washed with brine (3×30 mL), dried over MgSO_4 , concentrated *in vacuo* and recrystallised from EtOAc to afford 1.2 g (5.46 mmol, 83%) of the title compound as white needles; **m.p.** 120–121 °C (sublimed) (from EtOAc) [lit.,¹⁸¹ 146–148 °C (from CHCl_3 –hexane)]; **R_f** 0.22 (50:50:1 ethyl acetate – petroleum ether – acetic acid); $\nu_{\text{max}}/\text{cm}^{-1}$ 3340 (m, br, O–H and N–H), 2980 (w, C–H), 2938 (m, Cy C–H), 1680 (vs, C=O), 1524 (vs), [1167, 1067] (s, C–O); δ_{H} (400 MHz, CDCl_3) 4.35 (1H, s, H-2), 3.70 – 3.12 (2H, m, H-3 and H-6), 2.10 – 1.89 (4H, m, H-4, H-5, H-7 and H-8), 1.43 (9H, s, H-11, H-12 and H-13), 1.40 – 1.08 (4H, m, H-4, H-5, H-7 and H-8); δ_{C} (101 MHz, CDCl_3) 177.1 (s, C-1), 79.3 (s, C-10), 69.9 (d, C-6), 48.9 (d, C-3), 34.0 (t, C-5 and C-7), 31.2 (t, C-4 and C-8), 28.4 (q, C-11, C-12 and C-13); m/z (CI) (rel. int.) 234 (10), 233 (MNH_4^+ , 90), 216 (15), 177 (40), 148 (100) [Found MNH_4^+ , 233.1876. $\text{C}_{11}\text{H}_{21}\text{NO}_3$ requires MNH_4^+ , 233.1865; $\Delta = 4.7$ ppm].

3.2.5. Compound **52**⁵⁰Name: 3-(Chloromethyl)-6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazole .xHClChemical formula: C₈H₁₁ClN₂S

Exact mass: 202.0331

Molecular weight of M.HCl: 239.17



The compound was synthesised following a modified literature procedure.⁵⁰ To a flame-dried flask containing a solution of cyclic thiourea **90** (1.00 g, 7.69 mmol, 1.0 equiv.) in anhydrous MeCN (25 mL) was added 1,3-dichloroacetone **91** (1.00 g, 7.69 mmol, 1.0 equiv.) and the reaction mixture was allowed to stir under an atmosphere of N₂ at r.t. for 30 min until a white precipitate formed. The reaction mixture was then heated to 110 °C for 3 h, before allowing the temperature to cool down to r.t. The resulting white solid was collected by suction filtration, rinsed with dry MeCN and dried *in vacuo* to afford 1.44 g (6.01 mmol, 78%) of the title compound as a white amorphous powder; $\nu_{\text{max}}/\text{cm}^{-1}$ 3474 (w), 3409 (w), 2999 (m), 2986, 2855, 2759, 1554 (vs), 1486 (m), 811 (m), 698 (s), 645 (s); δ_{H} (400 MHz, DMSO-*d*₆) 10.44 (1H, s, H-5), 7.07 (1H, s, H-1), 4.85 (2H, s, H-9), 4.22 (2H, s, H-3), 1.51 (6H, s, H-7 and H-8); δ_{C} (101 MHz, DMSO-*d*₆) 206.9 (s, C-6), 111.5 (s, C-2), 110.0 (d, C-1), 70.0 (s, C-4), 58.3 (t, C-3), 36.3 (t, C-9), 27.8 (q, C-7 and C-8); *m/z* (ESI) (rel. int.) 224 (10), 205 (40), 203 (MH⁺, 100), 196 (10) [Found MH⁺, 203.0412. C₈H₁₁ClN₂S requires MH⁺, 203.0410; Δ = 1.0 ppm].

Experimental procedures

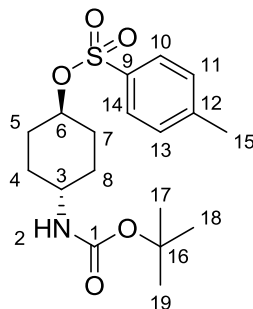
3.2.6. Compound **57**¹⁸³

Name: *tert*-Butyl *N*-{*trans*-4-[(4-methylbenzenesulfonyl)oxy]cyclohexyl} carbamate

Chemical formula: C₁₈H₂₇NO₅S

Exact mass: 369.1610

Molecular weight: 369.48



The compound was synthesised following a modified literature procedure.¹⁷⁸ To a solution of alcohol **47** (800 mg, 3.76 mmol, 1.0 equiv.) in anhydrous pyridine (8 mL) under an atmosphere of N₂ was added at 0 °C TsCl (1.50 g, 7.85 mmol, 2.1 equiv.). The reaction mixture was allowed to stir at 0 °C for 2 h and was concentrated *in vacuo*. The residue was taken up in EtOAc and washed with 0.5 N HCl_(aq) (4×20 mL). The organic layer was washed with brine (1×20 mL), dried over MgSO₄ and concentrated *in vacuo*. The white residue was purified by flash column chromatography to afford 793 mg (2.15 mmol, 57%) of the title compound as a white amorphous powder; **R_f** 0.05 (50% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3403 (m, N–H), 2978 (w, C–H), 1698 (vs, C=O), 1513 (s), 1170 (vs, O=S=O), cm^{-1} ; δ_{H} (400 MHz, DMSO-*d*₆) 7.82 (2H, d, *J* = 8.3 Hz, H-10 and H-14), 7.49 (2H, d, *J* = 8.0 Hz, H-11 and H-13), 6.66 (1H, d, *J* = 7.1 Hz, H-2), 4.38 (1H, tt, *J* = 10.5, 3.9 Hz, H-6), 3.24 (1H, s, H-3), 2.44 (3H, s, H-15), 1.88 – 1.70 (4H, m, H-4, H-5, H-7 and H-8), 1.57 – 1.41 (2H, m, H-5 and H-7), 1.37 (9H, s, H-17, H-18 and H-19), 1.32 – 1.11 (2H, m, H-4 and H-8); δ_{C} (101 MHz, CDCl₃) 155.1 (s, C-1), 144.6 (s, C-12), 134.5 (s, C-9), 129.8 (d, C-11 and C-13), 127.6 (d, C-10 and C-14), 80.2 (d, C-6), 79.5 (s, C-16), 47.9 (d, C-3), [30.7, 30.5] (t, C-4, C-5, C-7 and C-8), 28.4 (q, C-17, C-18 and C-19), 21.7 (q, C-15); **m/z** (CI) (rel. int.) 387 (MNH₄⁺, 10), 233 (15), 148 (100) [Found MNH₄⁺, 387.1960. C₁₈H₂₇NO₅S requires M NH₄⁺, 387.1954; Δ = 1.5 ppm].

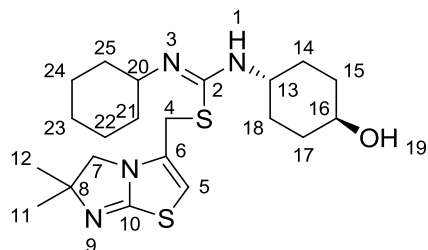
3.2.7. Compound **58**

Name: *N*'-Cyclohexyl-*N*-[(1*r*,4*r*)-4-hydroxycyclohexyl][({6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl}methyl)sulfanyl]methanimidamide .xHCl

Chemical formula: C₂₁H₃₄N₄OS₂

Exact mass: 422.2174

Molecular weight of M.2HCl: 495.57



A sealed oven-dried pressure vial containing a mixture of chloride **52** (239 mg, 1.00 mmol, 1.0 equiv.) and thiourea **59** (256 mg, 1.00 mmol, 1.0 equiv.) in anhydrous MeCN (10 mL) was allowed to stir at 90 °C overnight. The resulting precipitate was filtered hot *in vacuo* and the cake was washed with boiling MeCN (2×10 mL) to afford 330 mg (0.67 mmol, 67%) of the title compound as a white amorphous powder; $\nu_{\text{max}}/\text{cm}^{-1}$ 3360 (m, br, O-H), [2931, 2858] (m, C-H), 1607 (s, C=N), 1551 (s); δ_{H} (400 MHz, MeOH-*d*₄) 6.94 (1H, s, H-5), 4.67 (2H, s, H-4), 4.39 (2H, s, H-7), 3.84 (2H, br s, H-13 and H-20), 3.59 (1H, br s, H-16), 2.13 – 1.67 (11H, m, H-14, H-15, H-17, H-18, H-21, H-22, H-23, H-24 and H-25), 1.64 (6H, s, H-11 and H-12), 1.58 – 1.10 (7H, m, H-14, H-18, H-21, H-22, H-23, H-24 and H-25); δ_{C} (101 MHz, MeOH-*d*₄) 169.1 (s, C-2), 161.8 (s, C-10), 131.8 (s, C-6), 110.1 (d, C-5), 69.9 (s, C-8), 68.3 (d, C-16), 58.3 (t, C-7), [57.3, 56.3] (d, C-20), [54.1, 53.1] (d, C-13), [33.2, 33.0, 32.4, 31.4, 30.2, 29.3] (t, C-14, C-15, C-17, C-18, C-21 and C-25), 28.7 (t, C-4), 26.7 (q, C-11 and C-12), 24.8 (t, C-23), 24.5 (t, C-22 and C-24); m/z (ESI) (rel. int.) 425 (15), 424 (30), 423 (MH⁺, 100), 201 (25) [Found MH⁺, 423.2249. C₂₁H₃₄N₄OS₂ requires MH⁺, 423.2252; Δ = – 0.7 ppm].

Experimental procedures

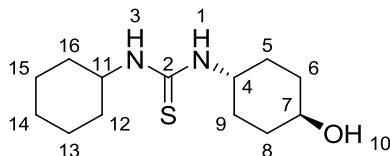
3.2.8. Compound **59**

Name: 3-Cyclohexyl-1-[*trans*-4-hydroxycyclohexyl]thiourea

Chemical formula: C₁₃H₂₄N₂OS

Exact mass: 256.1609

Molecular weight: 256.41



The compound was synthesised following a modified literature procedure.¹⁸⁴ To a pressure vial charged with commercially available ammonium **56** (1.16 g, 7.7 mmol, 1.1 equiv.) was added K₂CO₃ (580 g, 4.2 mmol, 0.6 equiv.) and EtOH (7 mL) and the reaction mixture was allowed to stir at r.t. for 5 min. Commercially available CyNCS (1.00 mL, 7.0 mmol, 1.0 equiv.) was added, the vial was sealed and the reaction mixture was allowed to stir at 85 °C for 6 h before cooling down to room temperature. The reaction mixture was then filtered through Celite[®] and the cake was washed with EtOH (30 mL). The filtrate was concentrated *in vacuo* to afford 2.05 g (7.0 mmol, quantitative) of the title compound as a white foam; *R_f* 0.13 (50% ethyl acetate in petroleum ether); *v*_{max}/cm⁻¹ 3273 (m, N–H and O–H), 2932 (m, Cy C–H), 2853 (m, C–H), 1536 (vs, N–H), 1448 (m, O–H), 1327 (vs, C=S), 1077 (vs, C–O); *δ*_H (400 MHz, CDCl₃) 5.58 (1H, br s, H-1), 5.38 (1H, br s, H-3), 3.93 (1H, br s, H-11), 3.78 – 3.59 (3H, m, H-4, H-7 and H-10), 2.20 – 1.09 (18H, m, H-5, H-6, H-8, H-9, H-12, H-13, H-14, H-15 and H-16); *δ*_C (101 MHz, CDCl₃) 179.5 (s, C-2), 69.6 (d, C-7), 52.8 (d, C-11), 52.3 (d, C-4), 33.7 (t, C-6 and C-8), 32.8 (t, C-12 and C-16), 30.6 (t, C-5 and C-9), 25.4 (t, C-14), 24.7 (t, C-13 and C-15); *m/z* (ESI) (rel. int.) 259 (10), 258 (21), 257 (MH⁺, 100) [Found MH⁺, 257.1683. C₁₃H₂₄N₂OS requires MH⁺, 257.1688; Δ = 1.9 ppm].

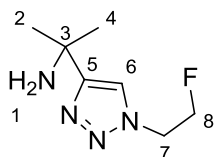
3.2.9. Compound **64**

Name: 2-[1-(2-Fluoroethyl)-1*H*-1,2,3-triazol-4-yl]propan-2-amine .HCl

Chemical formula: C₇H₁₃FN₄

Exact mass: 172.1124

Molecular weight of M.HCl: 208.66



Following the procedure for **65**, commercially available alkyne **62** (105 μ L, 1.00 mmol, 1.0 equiv.), FEA (**27**) (543 μ L at 1.84 M, 1.00 mmol, 1.0 equiv.), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (10.8 mg, 0.043 mmol, 4.3 mol%), sodium ascorbate (22.5 mg, 0.128 mmol, 12.8 mol%) and H_2O (543 μ L) yielded 60.3 mg (0.29 mmol, 29%) of the title compound as a yellow amorphous solid; $\nu_{\text{max}}/\text{cm}^{-1}$ 3388 (w, br, $\text{N}^+\text{--H}$), 2937 (m, C–H), 1517 (m), 1036 (m, C–F); δ_{H} (400 MHz, $\text{MeOH-}d_4$) 8.19 (1H, br s, H-6), 5.08 – 4.47 (7H, m, H-1, H-7 and H-8), 1.89 – 1.60 (6H, s, H-2 and H-4); δ_{C} (101 MHz, $\text{MeOH-}d_4$) 149.2 (s, C-5), 123.3 (d, C-6), 82.3 [t (d, $J = 170.7$ Hz), C-8], 51.6 [t (d, $J = 19.3$ Hz), C-7], 51.3 (s, C-3), 26.3 (q, C-2 and C-4); δ_{F} (377 MHz, $\text{MeOH-}d_4$) –224.4; m/z (ESI) (rel. int.) 174 (10), 173 (MH^+ , 80), 156 (100) [Found MH^+ , 173.1209. $\text{C}_7\text{H}_{13}\text{FN}_4$ requires MH^+ , 173.1202; $\Delta = 4.0$ ppm].

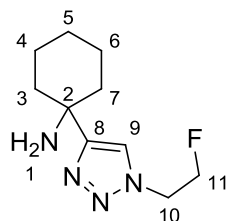
3.2.10. Compound **65**

Name: 1-[1-(2-Fluoroethyl)-1*H*-1,2,3-triazol-4-yl]cyclohexan-1-amine .HCl

Chemical formula: $\text{C}_{10}\text{H}_{17}\text{FN}_4$

Exact mass: 212.1437

Molecular weight of M.HCl: 248.73



The compound was synthesised following a modified literature procedure.¹⁸⁵ A mixture of commercially available alkyne **63** (135 μ L, 1.00 mmol, 1.0 equiv.), FEA (**27**) (543 μ L at 1.84 M in DMF, 1.00 mmol, 1.0 equiv.), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (10.8 mg, 0.043 mmol, 4.3 mol%) and sodium ascorbate (22.5 mg, 0.128 mmol, 12.8 mol%) was dissolved in water (543 μ L) and the reaction mixture was allowed to stir at r.t. for 1 h. The reaction mixture was then basified with 2 N $\text{NaOH}_{(\text{aq})}$ (10 mL) and extracted with CH_2Cl_2 (3 $\times$ 10 mL). The combined organic layers were washed with 2 N $\text{NaOH}_{(\text{aq})}$ (10 mL), treated dropwise with 2 N HCl in Et_2O (1 mL, 2.0 equiv.) and concentrated *in vacuo*. The residue was dispersed in Et_2O (50 mL) and centrifuged (5 min, 5 krpm, 4 $^\circ\text{C}$) and the supernatant was discarded. The pellet was dried under high vacuum overnight to afford 210 mg (0.84 mmol, 84%) of the title compound as a yellow foam; $\nu_{\text{max}}/\text{cm}^{-1}$ 3268 (m, br, $\text{N}^+\text{--H}$), 1638 (m), 1016 (m, C–F); δ_{H} (400 MHz, $\text{MeOH-}d_4$) 8.32 (1H, s, H-9), 5.04 – 4.63 (7H, m, H-1, H-10 and H-11), 2.52 (2H, br s, H-3 and H-7), 1.97 (2H, br s, H-3 and H-7), 1.76 (2H, br s, H-4 and H-6), 1.60 (1H, br s, H-5), 1.41 (3H, br

Experimental procedures

s, H-4, H-5 and H-6). δ_{C} (101 MHz, MeOH- d_4) 146.5 (s, C-8), 124.9 (d, C-9), 82.0 [t (d, $J = 170.4$ Hz), C-11], 54.1 (s, C-2), 51.3 [t (d, $J = 19.6$ Hz), C-10], 34.2 (t, C-3 and C-7), 24.5 (t, C-5), 21.8 (t, C-4 and C-6). δ_{F} (376 MHz, MeOH- d_4) -224.4 (tt, $J = 45.1, 22.6$ Hz); m/z (EI) 213 (5), 212 (M^+ , 30), 169 (80), 91 (100) [Found M^+ , 212.1443. $\text{C}_{10}\text{H}_{17}\text{FN}_4$ requires M^+ , 212.1437; $\Delta = 2.8$ ppm].

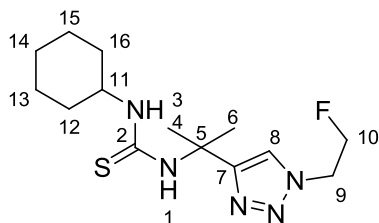
3.2.11. Compound **66**

Name: 1-Cyclohexyl-3-{2-[1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-yl]propan-2-yl}thiourea

Chemical formula: $\text{C}_{14}\text{H}_{24}\text{FN}_5\text{S}$

Exact mass: 313.1736

Molecular weight: 313.44

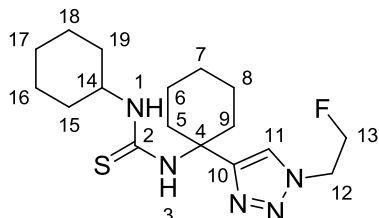


Following the procedure for **67**, ammonium chloride **64** (50.3 mg, 0.24 mmol, 1.0 equiv.), KHCO_3 (24.2 mg, 0.24 mmol, 1.0 equiv.), commercially available CyNCS (34.2 μL , 0.24 mmol, 1.0 equiv.) and EtOH (240 μL) afforded 36.7 mg (0.12 mmol, 49%) of the title compound as white amorphous solid after flash column chromatography; R_f 0.31 (65% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3380 (m), 3221 (m, br, N-H), 2934 (m, Cy C-H), [1540, 1516] (vs, N-H), 1232 (vs, C=S); δ_{H} (400 MHz, DMSO- d_6) 8.04 (1H, s, H-8), 7.44 (1H, s, H-1), 7.31 (1H, d, $J = 7.7$ Hz, H-3), 4.81 (2H, ddd, $J = 47.1, 5.2, 4.1$ Hz, H-10), 4.69 (2H, ddd, $J = 27.8, 5.3, 4.1$ Hz, H-9), 4.03 – 3.90 (1H, m, H-11), 1.83 – 1.75 (2H, m, H-12 and H-16), 1.73 (6H, s, H-4 and H-6), 1.70 – 1.42 (3H, m, H-13, H-14 and H-15), 1.34 – 1.00 (5H, m, H-12, H-13, H-14, H-15 and H-16); δ_{C} (101 MHz, DMSO- d_6) 180.1 (s, C-2), 153.1 (s, C-7), 122.8 (d, C-8), 82.3 [t (d, $J = 168.5$ Hz), C-10], 81.5 (s, C-5), 52.5 (d, C-11), 50.5 [t (d, $J = 19.7$ Hz), C-9], 32.5 (t, C-12 and C-16), 28.7 (q, C-4 and C-6), 25.7 (t, C-14), 24.6 (t, C-13 and C-15); δ_{F} (376 MHz, DMSO- d_6) -221.9 (tt, $J = 46.5, 28.2$ Hz); m/z (ESI) (rel. int.) 1002 ($M_3\text{Cu}^+$, 25), 689 ($M_2\text{Cu}^+$, 100), 314 ($M\text{H}^+$, 80) [Found $M\text{H}^+$, 314.1799. $\text{C}_{14}\text{H}_{24}\text{FN}_5\text{S}$ requires $M\text{H}^+$, 314.1815; $\Delta = -5.1$ ppm].

3.2.12. Compound **67**Name: 3-Cyclohexyl-1-{1-[1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-yl]cyclohexyl}thioureaChemical formula: C₁₇H₂₈FN₅S

Exact mass: 353.2049

Molecular weight: 353.50



The compound was synthesised following a modified literature procedure.¹⁸⁴ was modified as follows: to a mixture of ammonium chloride **65** (206 mg, 0.83 mmol, 1.0 equiv.) and KHCO₃ (83.0 mg, 0.83 mmol, 1.0 equiv.) in EtOH (830 μ L) was added commercially available CyNCS (117 μ L, 0.83 mmol, 1.0 equiv.) and the reaction mixture was allowed to stir at 35 °C overnight. The reaction mixture was then concentrated *in vacuo* and purified by flash column chromatography to afford 130 mg (0.37 mmol, 45%) of the title compound as a white amorphous solid; *R_f* 0.46 (65% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3428 (m, N–H), 1659 (w), 1051 (vs, C=S), [1024, 1004] (vs, C–F); δ_{H} (400 MHz, DMSO-*d*₆) 8.02 (1H, s, H-3), 7.52 (1H, d, *J* = 6.1 Hz, H-1), 7.10 (1H, s, H-11), 4.85 (2H, dt, *J* = 47.3, 4.6 Hz, H-13), 4.74 (2H, dt, *J* = 27.6, 4.6 Hz, H-12), 4.10 – 3.97 (1H, m, H-14), 2.50 – 2.39 (2H, m, H-5 and H-9), 2.04 – 1.98 (2H, m, H-5 and H-9), 1.95 – 1.79 (2H, m, H-15 and H-19), 1.79 – 1.21 (12H, m, H-6, H-7, H-8, H-16, H-17 and H-18), 1.21 – 1.04 (2H, m, H-15 and H-19); δ_{C} (101 MHz, CDCl₃) 179.0 (s, C-2), 151.5 (s, C-10), 122.1 (d, C-11), 81.3 [t (d, *J* = 173.0 Hz), C-13], 54.7 (d, C-14), 54.6 (s, C-4), 50.8 [t (d, *J* = 20.5 Hz), C-12], 35.5 (t, C-5 and C-9), 32.2 (t, C-15 and C-19), 25.4 (t, C-17), 25.2 (t, C-7), 24.4 (t, C-16 and C-18), 21.6 (t, C-6 and C-8); δ_{F} (376 MHz, CDCl₃) –75.3 (TFA), –221.7 (tt, *J* = 46.8, 26.9 Hz); *m/z* (ESI) (rel. int.) 1123 (M₃Cu⁺, 15), 772 (20), 771 (60), 770 (40), 769 (M₂Cu⁺, 100), 354 (MH⁺, 50) [Found MH⁺, 354.2108. C₁₇H₂₈FN₅S requires MH⁺, 354.2128; Δ = –5.6 ppm].

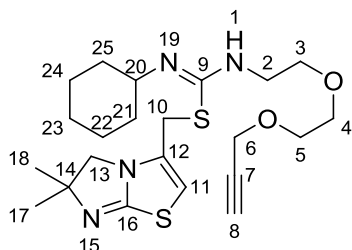
3.2.13. Compound **68**

Name: *N*-[(Cyclohexylimino)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methyl]-2-[2-(prop-2-yn-1-yloxy)ethoxy]ethan-1-amine .xTFA

Chemical formula: C₂₂H₃₄N₄O₂S₂

Exact mass: 450.2123

Molecular weight of M.3TFA: 792.73



Following the procedure for **106**, chloride **52** (441 mg, 1.84 mmol, 1.0 equiv.), thiourea **76** (524 mg, 1.84 mmol, 1.0 equiv.) and EtOH (18.4 mL) afforded 494 mg (0.624 mmol, 34%) of the title compound as a light yellow glass after purification by flash column chromatography followed by preparative HPLC, isocratic 15% B for 5 min and then gradient 15–80% B over 20 min; *R_f* 0.19 (25% methanol in acetone); *v*_{max}/cm^{−1} 3254 (w, br, N⁺–H), 2926 (m, Cy C–H), 2852 (m, C–H), 1632 (s, C=N⁺), [1592, 1560] (s, N⁺–H), 1305 (s), 1099 (vs, CH₂–O–CH₂); *δ*_H (400 MHz, CDCl₃) 9.55 (3H, br s, CF₃CO₂H), 4.93 (1H, d, *J* = 13.3 Hz, H-10), 4.38 (1H, d, *J* = 13.3 Hz, H-10), 4.36 (1H, d, *J* = 14.5 Hz, H-1), 4.19 (1H, d, *J* = 13.6 Hz, H-13), 4.13 (2H, dd, *J* = 2.2, 1.2 Hz, H-6), 3.88 (1H, d, *J* = 12.9 Hz, H-13), 3.80 – 3.48 (8H, m, H-2, H-3, H-4 and H-5), 3.19 (1H, tt, *J* = 11.2, 4.0 Hz, H-20), 2.52 (1H, t, *J* = 2.3 Hz, H-8), 1.99 – 1.76 (4H, m, H-21, H-22, H-24 and H-25), 1.70 – 1.57 (3H, m, H-22, H-23 and H-24), 1.55 (6H, s, H-17 and H-18), 1.36 – 1.08 (3H, m, H-21, H-23 and H-25); *δ*_C (101 MHz, CDCl₃) 171.3 (s, C-9), 166.5 (s, C-16), 161.5 [s (q, *J* = 35.6 Hz), CF₃CO₂H], 116.5 [s (q, *J* = 292.2 Hz), CF₃CO₂H], 88.5 (s, C-12), 79.5 (s, C-7), 75.3 (d, C-8), 71.6 (s, C-14), 70.4 (t, C-4), 68.8 (t, C-5), 66.9 (t, C-3), 62.0 (d, C-20), 58.4 (t, C-6), 56.0 (t, C-2), 43.8 (t, C-10), 36.0 (t, C-13), [32.2, 31.9] (t, C-22 and C-24), [28.2, 27.7] (t, C-17 and C-18), [25.1, 25.0] (t, C-21 and C-25), 24.7 (t, C-23); *δ*_F (376 MHz, CDCl₃) –75.48 (TFA); *m/z* (ESI) (rel. int.) 454 (5), 453 (10), 452 (30), 451 (MH⁺, 100), 369 (5), 291 (5), 247 (5) [Found MH⁺, 451.2217. C₂₂H₃₄N₄O₂S₂ requires MH⁺, 451.2201; Δ = 3.5 ppm].

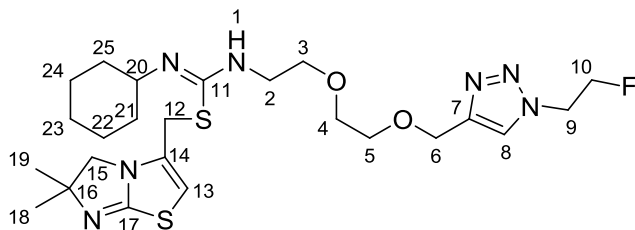
3.2.14. Compound **69**

Name: *N*-[(Cyclohexylimino)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methyl-2-(2-{[1-(2-fluoroethyl)-1*H*-1,2,3-triazol-4-yl]methoxy}ethoxy)ethane-1-amine .xTFA

Chemical formula: C₂₄H₃₈FN₇O₂S₂

Exact mass: 539.2512

Molecular weight of M.3TFA: 881.80



The compound was synthesised following a modified literature procedure.¹⁸⁵ Alkyne **68** (100 mg, 0.19 mmol, 1.0 equiv.) was taken up in a solution of FEA (**27**) in DMF (516 μ L, 1.84 M, 5.0 equiv.) and was diluted with 516 μ L of DMF and 1.03 mL of sodium acetate buffer (250 mM, pH 5). To this solution was added CuSO₄·5H₂O (9.6 mg, 0.038 mmol, 20 mol%) and sodium ascorbate (167 mg, 0.95 mmol, 5 equiv.) and the reaction mixture was allowed to stir at r.t. overnight and concentrated *in vacuo*. The residue was loaded on silica and purified by flash column chromatography followed by preparative HPLC, isocratic 15% B for 5 min then gradient 15–80% B over 20 min]. The collected fraction was freeze-dried to afford 21.3 mg (0.024 mmol, 13%) of the title compound as a colourless glass; **R_f** 0.40 (15% methanol in methylene chloride); δ_{H} (400 MHz, MeOH-*d*₄) 8.04 (1H, s, H-8), 4.91 – 4.76 (2H, dt, *J* = 47.6, 5.1 Hz, H-10), 4.82 – 4.71 (2H, dt, *J* = 26.8, 4.5 Hz, H-9), 4.65 (1H, s, H-6), [4.24, 3.87] (2H, d, *J* = 12.3 Hz, H-12), 3.83 – 3.49 (8H, m, H-2, H-3, H-4 and H-5), [3.27, 2.93] (2H, d, *J* = 8.8 Hz, H-6), 2.92 (1H, br s, H-20), 1.84 – 1.60 (5H, m, H-21, H-22, H-23, H-24 and H-25), 1.43 – 1.18 (5H, m, H-21, H-22, H-23, H-24 and H-25), [1.30, 1.29] (6H, s, H-18 and H-19); δ_{C} (101 MHz, CDCl₃) 188.0 (s, C-17), 171.5 (s, C-11), 144.6 (s, C-7), 124.2 (d, C-8), 118.0 (d, C-13), 88.1 (s, C-14), 81.7 [t (d, *J* = 172.3 Hz), C-10], 71.5 (s, C-16), [70.4, 69.5, 66.8] (t, C-3, C-4 and C-5), 64.0 (d, C-20), 62.4 (t, C-6), 56.2 (t, C-15), 50.8 [t (d, *J* = 20.2 Hz), C-9], 46.0 (t, C-2), 44.0 (t, C-12), [32.3, 32.0] (t, C-21 and C-25), [28.3, 27.8] (q, C-18 and C-19), [25.1, 25.0] (t, C-22 and C-24), 24.8 (t, C-23); δ_{F} (376 MHz, CDCl₃) – 75.6 (s, TFA), –222.1 (tt, *J* = 46.6, 27.3 Hz, –CH₂CH₂F); **m/z** (ESI) (rel. int.) 542 (15), 541 (35), 540 (MH⁺, 100) [Found MH⁺, 540.2584. C₂₄H₃₈FN₇O₂S₂ requires MH⁺, 540.2591; Δ = – 1.3 ppm].

Experimental procedures

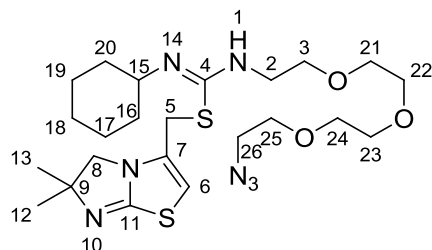
3.2.15. Compound **70**

Name: 2-{2-[2-(2-Azidoethoxy)ethoxy]ethoxy}-*N*-[(cyclohexylimino)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methyl]ethan-1-amine .xTFA

Chemical formula: C₂₃H₃₉N₇O₃S₂

Exact mass: 525.2556

Molecular weight of M.3TFA: 867.80



Following the procedure for **106**, chloride **52** (59.8 mg, 0.25 mmol, 1.0 equiv.), thiourea **79** (89.8 mg, 0.25 mmol, 1.0 equiv.) and anhydrous MeCN (2.5 mL) afforded 105 mg (0.12 mmol, 48%) of the title compound as a light yellow oil after purification by preparative HPLC, gradient 20–40% B over 10 min; *R_f* 0.50 (50% methanol in ethyl acetate); *v*_{max}/cm⁻¹ [2934, 2863] (m, C–H), 2103 (m, N₃), 1672 (s, C=N); *δ*_H (400 MHz, MeOH-*d*₄) 6.96 (1H, d, *J* = 54.3 Hz, H-6), 4.52 (2H, d, *J* = 12.8 Hz, H-5), 4.30 (2H, s, H-8), 3.91 – 3.57 (15H, m, H-2, H-3, H-15, H-21, H-22, H-23, H-24 and H-25), 3.37 (2H, t, *J* = 4.9 Hz, H-26), 2.01 – 1.64 (5H, m, H-16, H-17, H-18, H-19 and H-20), 1.61 (6H, s, H-12 and H-13), 1.56 – 1.13 (5H, m, H-16, H-17, H-18, H-19 and H-20); *δ*_C (101 MHz, MeOH-*d*₄) 169.1 (s, C-4), [164.1, 162.6] (s, C-11), 160.9 [s (q, *J* = 35.4 Hz), CF₃CO₂H], [132.0, 131.7] (s, C-7), 116.5 [s (q, *J* = 292.0 Hz), CF₃CO₂H], [110.2, 109.8] (d, C-6), [70.2, 70.1, 70.0, 69.9, 69.6] (t, C-21, C-22, C-23, C-24 and C-25), [68.5, 68.4] (s, C-9), 58.0 (t, C-8), 56.5 (d, C-15), 54.0 (t, C-3), 50.4 (t, C-26), [46.5, 44.7] (t, C-2), [32.4, 31.3] (t, C-16 and C-20), [28.4, 27.9] (t, C-5), 26.7 (q, C-12 and C-13), [24.51, 24.47, 24.4] (t, C-17, C-18 and C-19); *δ*_F (376 MHz, MeOH-*d*₄) – 76.9 (s, TFA); *m/z* (ESI) (rel. int.) 528 (10), 527 (25), 526 (MH⁺, 80), 391 (100) [Found MH⁺, 526.2646. C₁₂H₃₉N₇O₃S₂ requires MH⁺, 526.2634; Δ = 2.3 ppm].

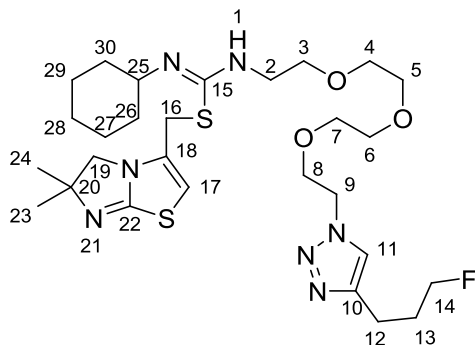
3.2.16. Compound **71**

Name: *N*-[(cyclohexylimino)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*]thiazol-3-yl)methyl)sulfanylmethyl]-2-[2-(2-{2-[4-(3-fluoropropyl)-1*H*-1,2,3-triazol-1-yl]ethoxy}ethoxy)ethoxy]ethan-1-amine .xTFA

Chemical formula: C₂₈H₄₆FN₇O₃S₂

Exact mass: 611.3088

Molecular weight of M.3TFA: 953.91



Following the procedure for **69**, alkyne **40** (379 μ L, 1.52 M in CH₂Cl₂, 580 μ mol, 10 equiv.), azide **70** (50.0 mg, 58 μ mol, 1.0 equiv.), MeOH (100 μ L), PBS (100 μ L), CuSO₄·5H₂O (2.9 mg, 12 μ mol, 20 mol%) and sodium ascorbate (51.0 mg, 290 μ mol, 5.0 equiv.) afforded 17.1 mg (18 μ mol, 31%) of the title compound as a colourless glass after purification by preparative HPLC, gradient 5–45% B over 10 min; $\nu_{\text{max}}/\text{cm}^{-1}$ 3415 (w, br, N⁺–H), 2937 (w, C–H), 1677 (C=N), 1200 (C–F); δ_{H} (400 MHz, MeOH-*d*₄) 7.88 – 7.83 (1H, m, H-11), 7.09 – 6.77 (1H, m, H-17), 4.68 – 4.36 (6H, m, H-9, H-14 and H-16), 4.06 – 3.53 (16H, m, H-2, H-3, H-4, H-5, H-6, H-7, H-8 and H-19), 3.52 – 3.39 (1H, m, H-25), 2.88 – 2.81 (2H, m, H-12), 2.19 – 1.69 (7H, m, H-13, H-26, H-27, H-28, H-29 and H-30), 1.68 – 1.57 (6H, m, H-23 and H-24), 1.56 – 1.12 (5H, m, H-26, H-27, H-28, H-29 and H-30); δ_{C} (101 MHz, MeOH-*d*₄) 172.0 (s, C-15), [169.8, 169.1] (s, C-22), 164.0 [s (d, *J* = 2.7 Hz), C-10], 131.9 (s, C-18), 122.7 (d, C-11), 110.2 (d, C-17), 82.5 [t (d, *J* = 164.1 Hz), C-14], [70.9, 70.4, 70.2, 70.1, 69.9, 69.8, 68.4] (t, C-4, C-5, C-6 and C-7), [69.0, 68.9] (s, C-20), 67.5 (t, C-8), 60.4 (d, C-25), [58.0, 56.6] (t, C-19), 55.4 (t, C-3), 49.9 (t, C-9), [45.3, 44.0] (t, C-2), [32.4, 31.7, 31.3] (t, C-26 and C-30), 29.9 [t (d, *J* = 20.0 Hz), C-13], [28.4, 28.0] (t, C-16), [26.7, 26.6, 26.4] (q, C-23 and C-24), 24.5 (t, C-28), 24.4 (t, C-27 and C-29), [20.9, 20.82, 20.79, 20.76] (t, C-12); δ_{F} (376 MHz, MeOH-*d*₄) –77.0 (TFA), –221.9 (sym. m); *m/z* (ESI) (rel. int.) 614 (10), 613 (20), 612 (MH⁺, 55), 311 (100) [Found MH⁺, 612.3150. C₂₈H₄₆FN₇O₃S₂ requires MH⁺, 611.3166; Δ = –2.6 ppm].

Experimental procedures

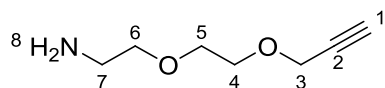
3.2.17. Compound **72**¹⁸⁶

Name: 3-[2-(2-Aminoethoxy)ethoxy]prop-1-yne

Chemical formula: C₇H₁₃NO₂

Exact mass: 143.0946

Molecular weight: 143.18



A solution of carbamate **75** (189 mg, 0.78 mmol, 1.0 equiv.) in CH₂Cl₂ (2 mL) was treated with TFA (2 mL) and the reaction mixture was allowed to stir for 45 min at r.t. and then concentrated *in vacuo*. The residue was taken up in CH₂Cl₂, K₂CO₃ was added and the mixture was allowed to stir at r.t. overnight before drying over Na₂SO₄ and concentrating *in vacuo* to afford 89.7 mg (0.63 mmol, 80%) of the title compound as a fluent yellow oil; **R_f** 0.04 (25% methanol in ethyl acetate); **δ_H** (400 MHz, CDCl₃) 4.21 (2H, d, *J* = 2.4 Hz, H-3), 3.73 – 3.63 (4H, m, H-4 and H-5), 3.51 (2H, t, *J* = 5.2 Hz, H-6), 2.87 (2H, br t, *J* = 5.2 Hz, H-7), 2.43 (1H, t, *J* = 2.4 Hz, H-1), 1.63 (2H, br s, H-8); **m/z** (CI) (rel. int.) 146 (5), 145 (25), 144 (MH⁺, 100), 107 (25) [Found MH⁺, 144.1024. C₇H₁₃NO₂ requires MH⁺, 144.1025; Δ = – 0.7 ppm].

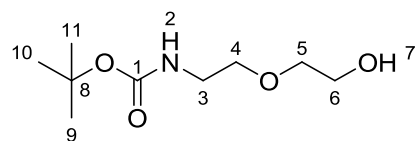
3.2.18. Compound **74**¹⁸⁷

Name: *tert*-Butyl *N*-[2-(2-hydroxyethoxy)ethyl]carbamate

Chemical formula: C₉H₁₉NO₄

Exact mass: 205.1314

Molecular weight: 205.25



To a solution of commercially available amine **73** (2.43 g, 23 mmol, 1.0 equiv.) in 0.25 M NaOH_(aq) – dioxane (1:1) (80 mL) chilled at 0 °C was added Boc₂O (5.35 g, 30.7 mmol, 1.34 equiv.) in one portion, the reaction mixture was allowed to stir for 30 min at 0 °C and for 24 h at room temperature. Dioxane was removed *in vacuo* and the reaction mixture was acidified to pH 3 with 2 N HCl_(aq) and extracted with EtOAc (3×100 mL). The combined organic layers were dried over Na₂SO₄ and concentrated *in vacuo* to afford 4.72 g (23 mmol, quantitative) of the title compound as a colourless oil; **ν_{max}**/cm^{–1} 3343 (w, br, O-H), [2976,

2933] (w, C–H), 1687 (vs, C=O), 1168 (vs), 1122 (vs, –O–), 1064 (s); δ_{H} (400 MHz, CDCl_3) 5.15 (1H, br s, H-2), 3.73 – 3.68 (2H, m, H-6), 3.58 – 3.47 (4H, m, H-4 and H-5), 3.34 – 3.24 (2H, m, H-3), 2.78 (1H, br s, H-7), 1.41 (9H, s, H-9, H-10 and H-11); δ_{C} (101 MHz, CDCl_3) 156.2 (s, C-1), 79.3 (s, C-8), 72.2 (t, C-5), 70.3 (t, C-4), 61.6 (t, C-6), 40.4 (t, C-3), 28.4 (q, C-9, C-10 and C-11); m/z (ESI) (rel. int.) 328 (10), 269 (20), 229 (10), 228 (MNa^+ , 100) [Found MNa^+ , 228.1219. $\text{C}_9\text{H}_{19}\text{NO}_4$ requires MNa^+ , 228.1212; $\Delta = 3.1$ ppm].

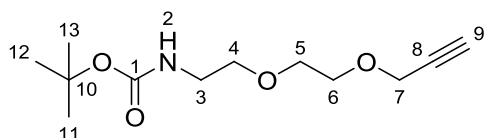
3.2.19. Compound **75**¹⁸⁷

Name: *tert*-Butyl *N*-{2-[2-(prop-2-yn-1-yloxy)ethoxy]ethyl} carbamate

Chemical formula: $\text{C}_{12}\text{H}_{21}\text{NO}_4$

Exact mass: 243.1471

Molecular weight: 243.30



To a solution of alcohol **74** (3.56 g, 17.4 mmol, 1.0 equiv.), TBAI (356 mg, 0.96 mmol, 0.055 equiv.), KI (356 mg, 2.14 mmol, 0.12 equiv.) and propargyl bromide (80% in toluene, 3.30 mL, 29.6 mmol, 1.7 equiv.) in THF (71 mL) was added KOH (1.02 g, 18.2 mmol, 1.05 equiv.). The reaction mixture was then allowed to stir at r.t. overnight and concentrated *in vacuo*. The residue was partitioned between water (50 mL) and EtOAc (50 mL) and the organic layer was washed with 10% (w/w) $\text{NaHCO}_3(\text{aq})$ (50 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by flash column chromatography to afford 1.96 g (8.08 mmol, 46%) of the title compound as a fluent light yellow oil; R_f 0.30 (10% ethyl acetate in methylene chloride); $\nu_{\text{max}}/\text{cm}^{-1}$ 3291 (w, br, N–H), 2977 (w), 2933 (w), 2119 (w, $\text{C}\equiv\text{C}$), 1692 (s, C=O), 1165 (s), 1092 (s); δ_{H} (400 MHz, CDCl_3) 4.99 (1H, br s, H-2), 4.19 (2H, d, $J = 2.3$ Hz, H-7), 3.73 – 3.56 (4H, m, H-5 and H-6), 3.52 (2H, t, $J = 5.1$ Hz, H-4), 3.30 (2H, dt, $J = 5.04, 4.91$ Hz, H-3), 2.43 (1H, t, $J = 2.4$ Hz, H-9), 1.42 (9H, s, H-11, H-12 and H-13); δ_{C} (101 MHz, CDCl_3) 156.1 (s, C-1), 79.6 (s, C-8), 79.3 (s, C-10), 74.8 (d, C-9), [70.4, 70.2] (t, C-5 and C-6), 69.1 (t, C-4), 58.5 (t, C-7), 40.5 (t, C-3), 28.5 (q, C-11, C-12 and C-13); m/z (ESI) (rel. int.) 267 (15), 266 (MNa^+ , 100), 210 (10) [Found MNa^+ , 266.1379. $\text{C}_{12}\text{H}_{21}\text{NO}_4$ requires MNa^+ , 266.1368; $\Delta = 4.1$ ppm].

Experimental procedures

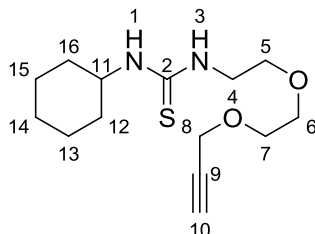
3.2.20. Compound 76

Name: 1-Cyclohexyl-3-{2-[2-(prop-2-yn-1-yloxy)ethoxy]ethyl}thiourea

Chemical formula: C₁₄H₂₄N₂O₂S

Exact mass: 284.1558

Molecular weight: 284.42



Following the procedure for **88**, CyNCS (683.2 mg, 4.84 mmol, 1.0 equiv.), amine **72** (691.7 mg, 4.84 mmol, 1.0 equiv.) and anhydrous MeCN (4.84 mL) afforded 1.22 mg (4.31 mmol, 89%) of the title compound as a thick yellow oil after purification by flash column chromatography; *R_f* 0.24 (50% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3259 (m, br, N–H), 2928 (m, Cy C–H), 2853 (m, C–H), 2114 (w, C≡C), 1541 (s, N–H), 1342 (s, C=S), 1095 (vs, CH₂–O–CH₂); δ_{H} (400 MHz, CDCl₃) 6.54 (2H, br s, H-1 and H-3), 4.16 (2H, d, *J* = 2.4 Hz, H-8), 4.06 – 3.73 (1H, m, H-11), 3.73 – 3.52 (8H, m, H-4, H-5, H-6 and H-7), 2.44 (1H, t, *J* = 2.4 Hz, H-10), 2.04 – 1.90 (m, H-13 and H-15), 1.74 – 1.63 (2H, m, H-12 and H-16), 1.63 – 1.53 (1H, m, H-14), 1.42 – 1.26 (2H, m, H-13 and H-15), 1.26 – 1.06 (3H, m, H-12, H-14 and H-16); δ_{C} (101 MHz, CDCl₃) 181.0 (s, C-2), 79.3 (s, C-9), 74.9 (d, C-10), [70.6, 70.1] (t, C-6 and C-7), 68.9 (t, C-5), 58.3 (t, C-8), 53.1 (d, C-11), 44.5 (t, C-4), 32.7 (t, C-12 and C-16), 25.5 (t, C-14), 24.8 (t, C-13 and C-15); *m/z* (EI) (rel. int.) 286 (5), 285 (10), 284 (*M*⁺, 40), 197 (100) [Found *M*⁺, 284.1554. C₁₄H₂₄N₂O₂S requires *MH*⁺, 284.1559; Δ = – 1.8 ppm].

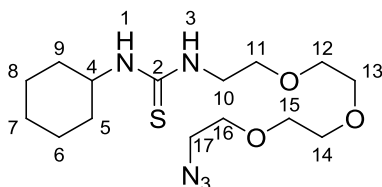
3.2.21. Compound 79

Name: 3-(2-{2-[2-(2-Azidoethoxy)ethoxy]ethoxy}ethyl)-1-cyclohexylthiourea

Chemical formula: C₁₅H₂₉N₅O₃S

Exact mass: 359.1991

Molecular weight: 359.49



The compound was synthesised following a modified literature procedure.¹⁸⁴ To a pressure vial was a solution of commercially available amine **77** (165 mg, 0.76 mmol, 1.0 equiv.) and CyNCS (107 mg, 0.76 mmol, 1.0 equiv.) in anhydrous MeCN (760 μ L). The pressure vial was sealed and the reaction mixture was allowed to stir at 90 °C overnight. The reaction mixture was then concentrated *in vacuo* to afford 263 mg (0.73 mmol, 96%) of the title compound as a white foam; R_f 0.65 (50% methanol in ethyl acetate); $\nu_{\max}/\text{cm}^{-1}$ 3284 (w, br), 2928 (m), 2855 (m), 2097 (s, N₃), 1542 (s, C=S), 1099 (vs); δ_{H} (400 MHz, CDCl₃) 6.52 (2H, br s, H-1 and H-3), 3.92 (1H, br s, H-4), 3.72 – 3.51 (14H, m, H-10, H-11, H-12, H-13, H-14, H-15 and H-16), 3.37 (2H, t, J = 5.0 Hz, H-17), 2.07 – 1.89 (2H, m, H-5 and H-9), 1.77 – 1.64 (2H, m, H-6 and H-8), 1.64 – 1.52 (1H, m, H-7), 1.45 – 1.25 (2H, m, H-5 and H-9), 1.24 – 1.04 (3H, m, H-6, H-7 and H-8); δ_{C} (101 MHz, CDCl₃) 181.2 (s, C-2), 72.4 (t, C-11), [70.6, 70.5, 70.4, 69.9] (t, C-12, C-13, C-14 and C-15), 61.6 (t, C-16), 53.0 (d, C-4), 50.6 (t, C-17), 44.5 (t, C-10), 32.8 (t, C-5 and C-9), 25.5 (t, C-7), 24.8 (t, C-6 and C-8); m/z (ESI) (rel. int.) 382 (MNa⁺, 30), 361 (15), 360 (MH⁺, 100) [Found MH⁺, 360.2078. C₁₅H₂₉N₅O₃S requires MH⁺, 360.2069; Δ = 2.5 ppm].

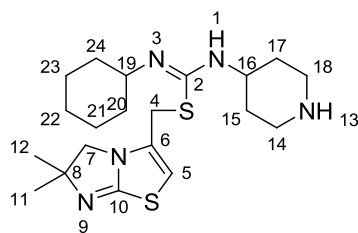
3.2.22. Compound **80**

Name: *N'*-Cyclohexyl-*N*-(piperidine-4-yl)[({6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl}methyl)sulfanyl]methanimidamide .xTFA

Chemical formula: C₂₀H₃₃N₅S₂

Exact mass: 407.2177

Molecular weight of M.4TFA: 863.73



A mixture of chloride **52** (239 mg, 1.00 mmol, 1.0 equiv.), thiourea **93** (341 mg, 1.00 mmol, 1.0 equiv.), KHCO₃ (200 mg, 2.00 mmol, 2.0 equiv.) in anhydrous MeCN (10 mL) was allowed to stir at 35 °C for 7 days. The reaction mixture was concentrated *in vacuo*, the residue taken up in saturated NaHCO_{3(aq)} (15 mL) and extracted with CH₂Cl₂ (3×15 mL). The combined organic layers were treated with TFA (1 mL) and this mixture was allowed to stir at r.t for 1 h. The mixture was quenched with saturated NaHCO_{3(aq)} and the product was extracted with CH₂Cl₂ (2×50 mL). The combined organic layers were concentrated *in vacuo*

Experimental procedures

and the resulting residue was taken up in 30 mL of 5% MeCN + 0.1% TFA in H₂O + 0.1% TFA. The dissolved material was purified by preparative HPLC, gradient 5–50% B over 20 min to afford 139 mg (0.27 mmol, 27%) of the title compound as a white foam; $\nu_{\max}/\text{cm}^{-1}$ [2940, 2863] (w, C–H), 1666 (s, C=N), 1591 (m); δ_{H} (400 MHz, MeOH-*d*₄) 6.94 (1H, s, H-5), 4.65 – 4.47 (2H, m, H-4), 4.33 (2H, s, H-7), 4.21 – 2.63 (10H, m, H-14, H-15, H-16, H-17, H-18 and H-19), 2.31 – 1.65 (8H, m, H-13, H-20, H-21, H-22, H-23 and H-24), 1.63 – 1.55 (6H, m, H-11 and H-12), 1.44 – 1.16 (3H, m, H-21, H-22 and H-23); δ_{C} (101 MHz, MeOH-*d*₄) 169.1 (s, C-2), [162.6, 162.3] (s, C-10), 160.3 [s (q, *J* = 37.5 Hz), CF₃CO₂H], 131.5 (s, C-6), 116.1 [s (q, *J* = 291.6 Hz), CF₃CO₂H], 110.1 (d, C-5), [70.8, 69.9] (s, C-8), [58.1, 58.0] (t, C-7), [57.5, 54.3] (d, C-19), [55.8, 53.1] (t, C-14 and C-18), [52.0, 49.3] (d, C-16), [44.1, 43.3, 43.1, 42.6] (t, C-15 and C-17), [32.2, 31.3, 31.1] (t, C-20 and C-24), [28.4, 28.2] (t, C-4), 27.4 (t, C-22), [26.8, 26.7, 26.6, 26.3] (q, C-11 and C-12), [24.7, 24.4, 24.31, 24.25] (t, C-21 and C-23); δ_{F} (376 MHz, MeOH-*d*₄) –77.2 (TFA); *m/z* (ESI) (rel. int.) 410 (5), 409 (30), 408 (MH⁺, 100), 325 (30), 201 (40) [Found MH⁺, 408.2259. C₂₀H₃₃N₅S₂ requires MH⁺, 408.2256; Δ = 0.7 ppm].

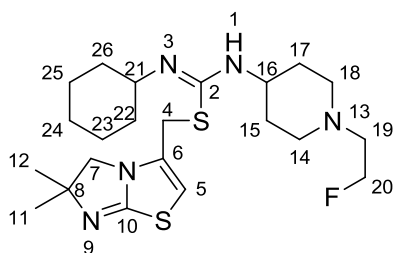
3.2.23. Compound 82

Name: *N'*-Cyclohexyl-*N*-[1-(2-fluoroethyl)piperidine-4-yl][(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfonyl]methanimidamide .xTFA

Chemical formula: C₂₂H₃₆FN₅S₂

Exact mass: 453.2396

Molecular weight of M.3TFA: 795.75



To an oven-dried flask containing a solution of piperidine **80** (26.1 mg, 0.05 mmol, 1.0 equiv.) in anhydrous MeCN (500 μ L) was added K₂CO₃ (35.2 mg, 0.255 mmol, 5.1 equiv.) and the mixture was allowed to stir at r.t. for 10 min. Tosylate **81** (10.9 mg, 0.05 mmol, 1.0 equiv.) was added to the reaction mixture, which was then allowed to stir at 35 °C for 2 days before quenching with 2 N HCl_(aq) (255 μ L, 10.2 equiv.) and diluting to 5 mL with H₂O + 0.1% TFA. The solution was purified by preparative HPLC, gradient 5–25% B over 20 min and the collected fraction was freeze-dried to afford 6.91 mg (0.012 mmol, 24%)

of the title compound as a white foam; $\nu_{\max}/\text{cm}^{-1}$ 2935 (w, C–H), 1676 (s, C=N), 1201 (s, C–F), 1131 (m); δ_{H} (400 MHz, MeOH- d_4) 4.85 – 3.41 (12H, m, H-4, H-7, H-14, H-16, H-18, H-19, H-20 and H-21), 3.25 – 2.82 (2H, m, H-14 and H-18), 2.32 – 1.64 (9H, m, H-15, H-17, H-22, H-23, H-24, H-25 and H-26), [1.58, 1.57] (6H, s, H-11 and H-12), 1.49 – 1.07 (5H, m, H-22, H-23, H-24, H-25 and H-26); δ_{C} (101 MHz, MeOH- d_4) 170.8 (s, C-2), 160.6 (s, C-10), [144.5, 144.2] (s, C-6), 115.2 (d, C-5), 77.7 [t (d, $J = 168.2$ Hz), C-20], 70.1 (s, C-8), 56.2 (t, C-7), [50.2, 50.1] (d, C-16), [44.49, 44.48] (d, C-21), 43.4 (t C-14 and C-18), [34.62, 34.59, 33.41, 33.38] (t, C-15 and C-17), 33.2 (t, C-4), [31.0, 29.8] (t, C-22 and C-26), [26.7, 26.4] (q, C-11 and C-12), [25.3, 25.2] (t, C-24), [24.7, 24.5] (t, C-23 and C-25); δ_{F} (376 MHz, MeOH- d_4) –77.1 (TFA), –222.7 (tt, $J = 46.4, 27.4$ Hz); m/z (ESI) (rel. int.) 456 (5), 455 (10), 454 (MH^+ , 40), 183 (100) [Found MH^+ , 454.2477. $\text{C}_{22}\text{H}_{36}\text{FN}_5\text{S}_2$ requires MH^+ , 454.2469; $\Delta = 1.8$ ppm].

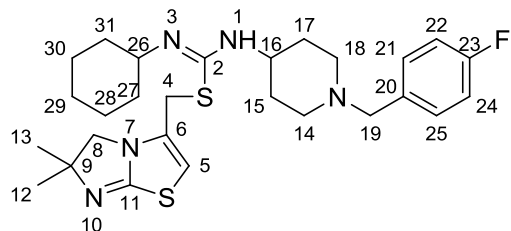
3.2.24. Compound 83

Name: *N'*-Cyclohexyl-*N*-{1-[(4-fluorophenyl)methyl]piperidin-4-yl}[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl]sulfanyl]methanimidamide .xHCl

Chemical formula: $\text{C}_{27}\text{H}_{38}\text{FN}_5\text{S}_2$

Exact mass: 515.2553

Molecular weight of M.4HCl: 661.60



To an oven-dried reaction vessel charged with a solution of chloride **52** (68.8 mg, 0.29 mmol, 1.0 equiv.) in anhydrous MeCN (2.9 mL) was added thiourea **88** (100 mg, 0.29 mmol, 1.0 equiv.). The vial was sealed and the reaction mixture allowed to stir at 35 °C for 7 days before concentrating *in vacuo*. The crude residue was purified by flash column chromatography and the collected fraction was concentrated *in vacuo*. The residue was dissolved in Et₂O (5 mL) and this solution was slowly acidified with 2 N HCl in Et₂O (2 mL). The resulting slurry was centrifuged (5 min, 5 krpm, 4 °C) and the pellet was dried under high vacuum to afford 33.8 mg (0.051 mmol, 18%) of the title compound as an amorphous off-white powder; R_f 0.26 (10% methanol in methylene chloride); $\nu_{\max}/\text{cm}^{-1}$ 3387 (s, br, $\text{N}^+\text{--H}$), 2935 (s, Cy C–H), 2870 (s, C–H), 1603 (vs, $\text{C}=\text{N}^+$), 1514 (s, $\text{N}^+\text{--H}$), 1228 (s, ar C–F); δ_{H}

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(500 MHz, MeOH- d_4) 7.79 – 7.07 (4H, m, H-21, H-22, H-24 and H-25), 4.71 – 3.04 (12H, m, H-4, H-8, H-14, H-16, H-18, H-19 and H-26), 2.71 – 1.19 (14H, m, H-15, H-16, H-27, H-28, H-29, H-30 and H-31), [1.13, 1.11] (6H, s, H-12 and H-13); δ_C (101 MHz, MeOH- d_4) 169.3 (s, C-2), 165.3 [s (d, $J = 249.1$ Hz), C-23], 135.0 [d (d, $J = 8.4$ Hz), C-21 and C-25], [133.6, 132.4] (s, C-6 and C-11), 129.9 (d, C-5), 126.3 [s (d, $J = 2.7$ Hz), C-20], 117.3 [d (d, $J = 22.0$ Hz), C-22 and C-24], 72.1 (s, C-9), 69.1 (t, C-14 and C-18), [61.0, 60.8] (t, C-8), [52.2, 52.1] (t, C-4), 46.7 (t, C-19), 40.2 (d, C-16), 31.6 (t, C-27 and C-31), 30.1 (t, C-15 and C-17), [29.5, 28.7, 28.0, 27.3] (d, C-26), 25.0 (t, C-29), 24.0 (t, C-28 and C-30), [14.4, 11.4] (q, C-12 and C-13); δ_F (376 MHz, MeOH- d_4) –112.5 (br s); m/z (ESI) (rel. int.) 519 (5), 518 (15), 517 (35), 516 (MH^+ , 100), 294 (45), 259 (MH_2^{2+} , 15) [Found MH^+ , 516.2617. $C_{27}H_{38}FN_5S_2$ requires MH^+ , 516.2631; $\Delta = -2.7$ ppm].

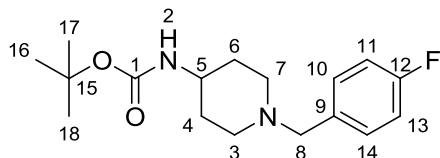
3.2.25. Compound **86**¹⁸⁸

Name: *tert*-Butyl *N*-{1-[(4-fluorophenyl)methyl]piperidine-4-yl}carbamate

Chemical formula: $C_{17}H_{25}FN_2O_2$

Exact mass: 308.1900

Molecular weight: 308.39



In a flame-dried flask, commercially available piperidine **84** (1.0 g, 5.0 mmol, 1.0 equiv.) was dissolved in anhydrous THF (50 mL) under an atmosphere of N_2 and chilled to 0 °C. To this solution were added NEt_3 (1.39 mL, 10.0 mmol, 2.0 equiv.) in one portion and commercially available bromide **85** (799 μ L, 6.5 mmol, 1.3 equiv.) dropwise. The reaction mixture was allowed to stir for 15 min at 0 °C and then for 7 h at r.t. and concentrated *in vacuo*. The residue was taken up in EtOAc (100 mL) and washed with water (100 mL) and brine (100 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to afford 1.47 g (4.77 mmol, 95%) of the title compound as an amorphous white powder; R_f 0.41 (10% methanol in methylene chloride); ν_{max}/cm^{-1} 3362 (m), 2935 (m), 2797 (m), 1678 (vs, C=O), 1521 (vs, aro), 1505 (vs, aro), 1288 (s), 1218 (s, C–F); δ_H (500 MHz, $CDCl_3$) 7.31 – 7.26 (2H, m, H-10 and H-14), 7.04 – 6.99 (2H, m, H-11 and H-13), 4.44 (1H, br s, H-2), 3.54 – 3.43 (1H, m, H-5), 3.47 (2H, br s, H-8), 2.84 – 2.75 (2H, m, H-3 and H-7), 2.16 – 2.05 (2H, m, H-3 and H-7), 1.98 – 1.88 (2H, m, H-4 and H-6), 1.49 – 1.39 (2H, m, H-4 and H-6), 1.47 (9H, s,

H-16, H-17 and H-18); δ_{C} (126 MHz, CDCl_3) 162.0 [d (d, $J = 244.9$ Hz), C-12], 155.2 (s, C-1), 130.6 (s, C-9), 130.5 [d (d, $J = 5.9$ Hz), C-10 and C-14], 115.0 [d (d, $J = 21.3$ Hz), C-11 and C-13], 79.3 (s, C-15), 62.2 (t, C-8), 52.2 (t, C-3 and C-7), 47.8 (d, C-5), 32.6 (t, C-4 and C-6), 28.4 (q, C-16, C-17 and C-18); δ_{F} (377 MHz, CDCl_3) -116.0 ; m/z (ESI) (rel. int.) 310 (20), 309 (MH^+ , 100), 253 (20) [Found MH^+ , 309.1971. $\text{C}_{17}\text{H}_{25}\text{FN}_2\text{O}_2$ requires MH^+ , 309.1978; $\Delta = -2.3$ ppm].

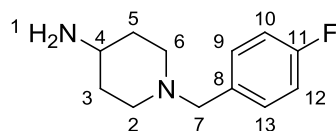
3.2.26. Compound **87**¹⁸⁸

Name: 1-[(4-Fluorophenyl)methyl]piperidin-4-amine

Chemical formula: $\text{C}_{12}\text{H}_{17}\text{FN}_2$

Exact mass: 208.1376

Molecular weight: 208.28



A solution of carbamate **86** (1.34 mg, 4.35 mmol) in CH_2Cl_2 (10 mL) was treated with TFA (10 mL) and the reaction mixture was allowed to stir at r.t. overnight and concentrated *in vacuo*. The residue was taken up in $\text{NaOH}_{(\text{aq})}$ (10 mL) and extracted with EtOAc (2×20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to afford 877.7 mg (4.22 mmol, 97%) of the title compound as thick deep yellow oil; $\nu_{\text{max}}/\text{cm}^{-1}$ 2933 (m, C-H), 1691 (m), 1603 (m), 1507 (vs), 1219 (vs, C-F); δ_{H} (400 MHz, CDCl_3) 7.31 – 7.25 (2H, m, H-9 and H-13), 7.04 – 6.97 (2H, m, H-10 and H-12), 3.47 (2H, s, H-7), 2.86 – 2.77 (2H, m, H-2 and H-6), 2.68 (1H, tt, $J = 10.5, 4.2$ Hz, H-4), 2.03 (2H, td, $J = 11.6, 2.6$ Hz, H-2 and H-6), 1.84 – 1.76 (2H, m, H-3 and H-5), 1.47 – 1.33 (2H, m, H-3 and H-5); δ_{C} (101 MHz, CDCl_3) 161.9 [s (d, $J = 224.7$ Hz), C-11], 134.3 (s, C-9), 130.5 [d (d, $J = 7.9$ Hz), C-9 and C-13], 114.9 [d (d, $J = 21.2$ Hz), C-10 and C-12], 62.2 (t, C-7), 52.3 (t, C-2 and C-6), 48.8 (d, C-4), 35.9 (t, C-3 and C-5); δ_{F} (376 MHz, CDCl_3) -75.5 (TFA), -116.1 (tt, $J = 11.2, 5.7$ Hz); m/z (ESI) (rel. int.) 317 (40), 210 (15), 209 (MH^+ , 100) [Found MH^+ , 209.1454. $\text{C}_{12}\text{H}_{17}\text{FN}_2$ requires MH^+ , 209.1454; $\Delta = 0.0$ ppm].

Experimental procedures

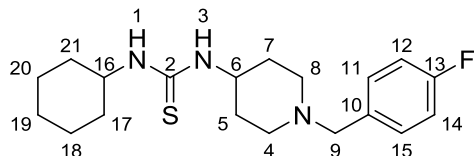
3.2.27. Compound **88**

Name: 1-(Cyclohexyl)-3-{1-[(4-fluorophenyl)methyl]piperidin-4-yl}thiourea

Chemical formula: C₁₉H₂₈FN₃S

Exact mass: 349.1988

Molecular weight: 349.51

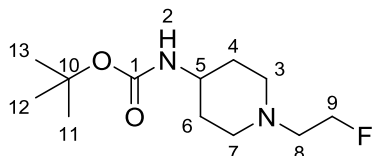


The compound was synthesised following a modified literature procedure.¹⁸⁴ To a sealed oven-dried pressure vial and under an atmosphere of N₂ was added a solution of CyNCS (347 mg, 2.46 mmol, 1.0 equiv.) and amine **87** (512 mg, 2.46 mmol, 1.0 equiv.) in anhydrous MeCN (2.5 mL). The reaction mixture was allowed to stir at 60 °C for 2 days before concentrating *in vacuo*. The residue was purified by flash column chromatography to afford 443 mg (1.27 mmol, 52%) of the title compound as an off-white amorphous powder; **R_f** 0.35 (10% methanol in methylene chloride); $\nu_{\text{max}}/\text{cm}^{-1}$ 3257 (w, br, N–H), 3061 (w, ar C–H), 2928 (m, Cy C–H), [1535, 1508] (vs, N–H), 1219 (vs, ar C–F); δ_{H} (400 MHz, MeOH-*d*₄) 7.40 – 7.32 (2H, m, H-11 and H-15), 7.11 – 7.04 (2H, m, H-12 and H-14), 4.26 – 3.99 (2H, m, H-6 and H-16), 3.57 – 3.49 (2H, s, H-9), 2.93 – 2.78 (2H, m, H-4 and H-8), 2.26 – 2.09 (2H, m, H-18 and H-20), 2.04 – 1.89 (4H, m, H-4, H-8, H-17 and H-21), 1.82 – 1.69 (2H, m, H-5 and H-7), 1.69 – 1.59 (1H, m, H-19), 1.59 – 1.46 (2H, m, H-18 and H-20), 1.46 – 1.31 (2H, m, H-5 and H-7), 1.31 – 1.11 (3H, m, H-17, H-19 and H-21); δ_{C} (101 MHz, MeOH-*d*₄) 176.0 (s, C-2), 162.7 [s (d, *J* = 226.5 Hz), C-13], 131.1 [d (d, *J* = 8.1 Hz), C-11 and C-15], 114.5 [d (d, *J* = 21.2 Hz), C-12 and C-14], 61.7 (t, C-9), 525.2 (d, C-16), 51.8 (t, C-4 and C-8), 50.5 (d, C-6), 32.4 (t, C-17 and C-21), 31.1 (t, C-18 and C-20), 25.3 (t, C-19), 24.6 (t, C-5 and C-7); δ_{F} (376 MHz, MeOH-*d*₄) –117.6; **m/z** (ESI) (rel. int.) 1111 (M₃Cu⁺, 15), 761 (M₂Cu⁺, 40), 352 (5), 351 (25), 350 (MH⁺, 100) [Found MH⁺, 350.2051. C₁₉H₂₈FN₃S requires MH⁺, 350.2066; Δ = –4.3 ppm].

3.2.28. Compound **89**Name: *tert*-Butyl-*N*-[1-(2-fluoroethyl)piperidine-4-yl]carbamateChemical formula: C₁₂H₂₃FN₂O₂

Exact mass: 246.1744

Molecular weight: 246.32



To an oven-dried flask equipped with a cooling condensor and containing a solution of commercially available piperidine **84** (400 mg, 2.00 mmol, 1 equiv.) in anhydrous DMF (20 mL) was added K₂CO₃ (276 mg, 2.00 mmol, 1.0 equiv.). The vial was sealed and the mixture was allowed to stir at r.t. for 10 min. Tosylate **81** (436 mg, 2.00 mmol, 1.0 equiv.) was added to the reaction mixture, which was then allowed to stir at r.t. for 10 min before being allowed to stir at 100 °C overnight and concentrated *in vacuo*. The residue was purified by flash column chromatography to afford 300 mg (1.22 mmol, 61%) of the title compound as a white amorphous solid; *R_f* 0.32 (10% methanol in methylene chloride); *v*_{max}/cm⁻¹ 3363 (m, N-H), 2975 (m, C-H) 1677 (vs, C=O), 1508 (vs, N-H), 1234 (s), 1159 (vs, C-F); *δ*_H (400 MHz, CDCl₃) 4.56 (2H, dt, *J* = 47.5, 4.8 Hz, H-9), 4.06 (2H, br s, H-3 and H-7), 2.95 (2H, dt, *J* = 28.1, 4.8 Hz, H-8), 2.82 (2H, t, *J* = 12.6 Hz, H-3 and H-7), 2.66 (1H, tt, *J* = 10.3, 3.8 Hz, H-5), 1.86 (2H, d, *J* = 11.8 Hz, H-4 and H-6), 1.47 (9H, s, H-11, H-12 and H-13), 1.36 – 1.20 (2H, m, H-4 and H-6); *δ*_C (101 MHz, CDCl₃) 154.8 (s, C-1), 83.8 [t (d, *J* = 165.2 Hz), C-9], 79.4 (s, C-10), 54.7 (d, C-5), 46.6 [t (d, *J* = 19.8 Hz), C-8], 42.5 (t, C-3 and C-7), 32.5 (t, C-4 and C-6), 28.4 (q, C-11, C-12 and C-13); *δ*_F (376 MHz, CDCl₃) – 223.73 (tt, *J* = 47.7, 28.5 Hz); *m/z* (ESI) (rel. int.) 248 (5), 247 (MH⁺, 25), 232 [MH₂(MeCN)(-*t*Bu)⁺, 30], 191 [MH₂(-*t*Bu)⁺, 100] [Found MH⁺, 247.1822. C₁₂H₂₃FN₂O₂ requires MH⁺, 247.1822; Δ = 0.0 ppm].

Experimental procedures

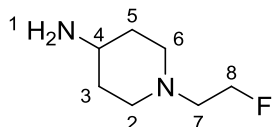
3.2.29. Compound **90**

Name: 4-Amino-1-(2-fluoroethyl)piperidine .xHCl

Chemical formula: C₇H₁₅FN₂

Exact mass: 146.1219

Molecular weight of M.2HCl: 219.13



A solution of carbamate **89** (300 mg, 1.22 mmol, 1.0 equiv.) in CH₂Cl₂ (4 mL) was treated dropwise with TFA (1 mL) at r.t. and the reaction mixture was allowed to stir for 1 h. The reaction was quenched by addition of saturated Na₂CO_{3(aq)} (15 mL) and the aqueous layer was extracted with CH₂Cl₂ (3×15 mL). The combined organic layers were dried over MgSO₄, acidified with 2 N HCl in Et₂O (1.5 mL, 2.5 equiv.) and concentrated *in vacuo*. The residue was dissolved in a minimum of MeOH (~1mL) and precipitated with Et₂O (50 mL). The resulting slurry was centrifuged (5 min, 5 krpm, 4 °C), the supernatant was discarded and the pellet resuspended in Et₂O (50 mL). Again, the slurry was centrifuged and the supernatant discarded. The pellet was dried under high vacuum to afford 265 mg (1.21 mmol, quantitative) of the title compound as a deep yellow amorphous powder; $\nu_{\text{max}}/\text{cm}^{-1}$ 3375 (w, br, N⁺-H), 2960 (w, C-H), 1624, 1051 (w, C-F); δ_{H} (400 MHz, CDCl₃) 4.55 (2H, dt, J = 47.5, 4.8 Hz, H-8), 3.16 (2H, dt, J = 13.1, 3.4 Hz, H-2 and H-6), 2.94 (2H, dt, J = 28.0, 4.8 Hz, H-7), 2.72 – 2.58 (3H, m, H-2, H-4 and H-6), 2.38 (2H, br s, H-1), 1.98 – 1.85 (2H, m, H-3 and H-5), 1.31 (2H, dddd, J = 13.2, 11.4, 10.3, 4.1 Hz, H-3 and H-5); δ_{C} (101 MHz, CDCl₃) 84.31 [t (d, J = 164.9 Hz), C-8], 54.5 (d, C-4), 46.5 [t (d, J = 19.4 Hz), C-7], 44.9 (t, C-2 and C-6), 33.4 (t, C-3 and C-5); δ_{F} (376 MHz, MeOH-*d*₄) –220.6; *m/z* (ESI) (rel. int.) 196 (20), 180 (20), 148 (10), 147 (MH⁺, 100) [Found MH⁺, 147.1302. C₇H₁₅FN₂ requires MH⁺, 147.1298; Δ = 2.7 ppm].

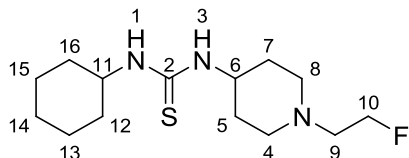
3.2.30. Compound **91**

Name: 1-(Cyclohexyl)-3-[1-(2-fluoroethyl)piperidin-4-yl]thiourea

Chemical formula: C₁₄H₂₆FN₃S

Exact mass: 287.1831

Molecular weight: 287.44



The compound was synthesised following a modified literature procedure.¹⁸⁴ To a solution of ammonium **90** (265 mg, 1.21 mmol, 1.0 equiv.) and KHCO₃ (254 mg, 2.54 mmol, 2.1 equiv.) in EtOH (1.2 mL) was added commercially available CyNCS (173 µL, 1.22 mmol, 1.0 equiv.) and the reaction mixture was allowed to stir at 35 °C overnight. The reaction mixture was then concentrated *in vacuo* and purified by flash column chromatography to afford 94.0 mg (0.327 mmol, 27%) of the title compound as a white foam; *R_f* 0.43 (50% methanol in ethyl acetate); *v*_{max}/cm⁻¹ 3282 (m, br), 2929 (m), 2862 (w), 1556 (vs, C=S), 1503 (s), 1235 (s), 1030 (s, C-F); *δ*_H (400 MHz, CDCl₃) 5.73 (1H, br s, H-3), 5.51 (1H, br s, H-1), 4.58 (2H, dt, *J* = 47.6, 4.9 Hz, H-10), 4.09 (1H, br s, H-6), 3.76 (1H, br s, H-11), 2.93 (2H, dt, *J* = 12.7, 3.4 Hz, H-4 and H-8), 2.72 (2H, dt, *J* = 28.3, 4.9 Hz, H-9), 2.28 (2H, td, *J* = 11.7, 2.6 Hz, H-4 and H-8), 2.19 – 1.94 (4H, m, H-5, H-7, H-12 and H-16), 1.76 (2H, dt, *J* = 13.6, 3.9 Hz, H-5 and H-7), 1.70 – 1.49 (3H, m, H-13, H-14 and H-15), 1.46 – 1.32 (2H, m, H-13 and H-15), 1.31 – 1.17 (3H, m, H-12, H-14 and H-16); *δ*_C (101 MHz, CDCl₃) 179.6 (s, C-2), 81.9 [t (d, *J* = 167.7 Hz), C-10], 58.1 [t (d, *J* = 19.9 Hz), C-9], 52.7 (d, C-6), 52.5 (t, C-4 and C-8), 51.1 (d, C-11), 32.8 (t, C-12 and C-16), 32.0 (t, C-5 and C-7), 25.4 (t, C-14), 24.6 (t, C-13 and C-15); *δ*_F (376 MHz, CDCl₃) –218.2 (tt, *J* = 49.8, 28.5 Hz); *m/z* (ESI) (rel. int.) 289 (20), 288 (MH⁺, 100) [Found MH⁺, 288.1906. C₁₄H₂₆FN₃S requires MH⁺, 288.1910; Δ = –1.4 ppm].

Experimental procedures

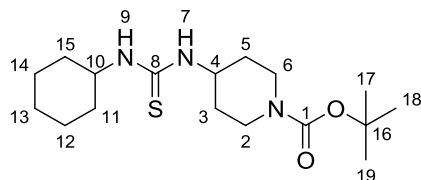
3.2.31. Compound **93**

Name: *tert*-Butyl 4-[(cyclohexylcarbamothioyl)amino]piperidine-1-carboxylate

Chemical formula: C₁₇H₃₁N₃O₂S

Exact mass: 341.2137

Molecular weight: 341.51



The compound was synthesised following a modified literature procedure.¹⁸⁴ To a solution of commercially available amine **92** (400 mg, 2.0 mmol, 1.0 equiv.) in anhydrous MeCN (2 mL) was added CyNCS (283 mg, 2.0 mmol, 1.0 equiv.) and the reaction mixture was allowed to stir at 35 °C for overnight. The reaction mixture was then concentrated *in vacuo* and purified by flash column chromatography to afford 684 mg (2.00 mmol, quantitative) of the title compound as a white foam; *R_f* 0.33 (50% ethyl acetate in petroleum ether); *v*_{max}/cm⁻¹ 3289 (w, br, N-H), 2929 (m, Cy C-H), 2852 (m, C-H), 1691 (m, C=O), 1536 (m, N-H), 1235 (s, C=S); *δ*_H (400 MHz, MeOH-*d*₄) 4.30 (1H, br s, H-4), 4.13 – 3.93 (3H, m, H-2, H-6 and H-10), 2.90 (2H, m, H-2 and H-6), 2.00 – 1.89 (4H, m, H-3, H-5, H-12 and H-14), 1.79 – 1.68 (2H, m, H-11 and H-15), 1.68 – 1.58 (1H, m, H-13), 1.46 (9H, s, H-17, H-18 and H-19), 1.41 – 1.30 (4H, m, H-3, H-5, H-12 and H-14), 1.26 – 1.14 (3H, m, H-11, H-13 and H-15); *δ*_C (101 MHz, MeOH-*d*₄) 177.0 (s, C-8), 155.1 (s, C-1), 79.7 (s, C-16), 51.8 (d, C-10), 50.2 (d, C-4), 42.2 (t, C-2 and C-6), 32.4 (t, C-11 and C-15), 31.4 (t, C-3 and C-5), 27.3 (q, C-17, C-18 and C-19), 25.3 (t, C-13), 24.6 (t, C-12 and C-14); *m/z* (ESI) (rel. int.) 1087 (M₃Cu⁺, 50), 745 (M₂Cu⁺, 100), 342 (MH⁺, 98), 286 (MH-*t*Bu⁺, 70) [Found MH⁺, 342.2200. C₁₇H₃₂N₃O₂S requires MH⁺, 342.2215; Δ = -4.4 ppm].

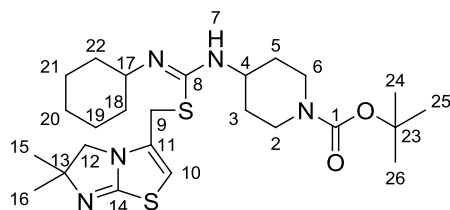
3.2.32. Compound **94**

Name: *tert*-Butyl 4-[[*(N'*-cyclohexylimino)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl]sulfanyl]methyl]amino}piperidine-1-carboxylate .xTFA

Chemical formula: C₂₅H₄₁N₅O₂S₂

Exact mass: 507.2702

Molecular weight of M.3TFA: 849.83



A mixture of chloride **52** (132 mg, 0.55 mmol, 1.0 equiv.), thiourea **93** (188 mg, 0.55 mmol, 1.0 equiv.), KHCO₃ (152 mg, 1.10 mmol, 2.0 equiv.) in anhydrous MeCN (5.5 mL) was allowed to stir at 35 °C for 7 days. The reaction mixture was concentrated *in vacuo* and the residue taken up in saturated NaHCO_{3(aq)} (15 mL) before extracting with CH₂Cl₂ (3×15 mL). The combined organic layers were concentrated *in vacuo* and the resulting residue was taken up in 30 mL of 5% MeCN + 0.1% TFA in H₂O + 0.1% TFA. The dissolved material was purified by preparative HPLC, gradient 5–50% B over 20 min to afford 229 mg (0.27 mmol, 50%) of the title compound as a white foam; *R_f* 0.64 (50% methanol in ethyl acetate); *ν*_{max}/cm⁻¹ 2938 (w, Cy C–H), 1675 (vs, C=O), 1590 (s), 1198 (vs), 1173 (vs), 1134 (vs); *δ*_H (400 MHz, CDCl₃) 4.41 – 3.91 (4H, m, H-2, H-4, H-6 and H-17), 3.91 – 3.34 (4H, m, H-9 and H-12), 2.86 – 2.65 (2H, dd, *J* = 12.0 Hz, H-2 and H-6), 2.63 – 2.24 (1H, br s, H-7), 1.91 – 1.61 (6H, m, H-3, H-5, H-18, H-19, H-21 and H-22), 1.61 – 1.36 (5H, m, H-3, H-5, H-19, H-20 and H-21), [1.59, 1.55] (6H, s, H-15 and H-16), [1.47, 1.46] (9H, s, H-24, H-25 and H-26), 1.36 – 1.08 (3H, m, H-18, H-20 and H-22); *δ*_C (101 MHz, CDCl₃) 189.0 (s, C-8), [164.1, 164.0] (s, C-14), [154.9, 154.8] (s, C-1), 148.8 (d, C-10), [83.6, 83.0] (s, C-11), [79.4, 79.2] (s, C-23), [74.7, 74.6] (s, C-13), 63.1 (d, C-4), 59.4 (d, C-17), [56.3, 56.2, 56.1] (t, C-2 and C-6), [42.5, 42.2] (t, C-9), [34.1, 34.0] (t, C-12), [29.6, 28.9, 28.8, 28.3, 26.8, 26.7, 26.0, 25.5] (t, C-3, C-5, C-18, C-19, C-20, C-21 and C-22), [29.3, 29.3] (q, C-15 and C-16), [28.5, 28.5] (q, C-24, C-25 and C-26); *δ*_F (376 MHz, MeOH-*d*₄) *δ* –77.2 (TFA); *m/z* (ESI) (rel. int.) 509 (25), 508 (MH⁺, 100), 452 (MH-*t*Bu⁺, 55), 408 (MH-Boc⁺, 15) [Found MH⁺, 508.2766. C₂₅H₄₁N₅O₂S₂ requires MH⁺, 508.2780; Δ = –2.8 ppm].

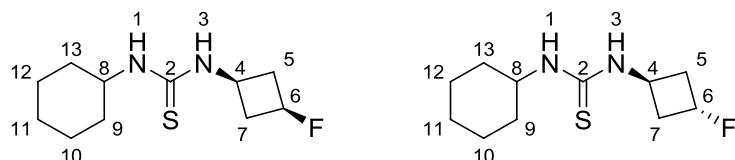
3.2.33. Compounds **96** and **98**

Name: 1-Cyclohexyl-3-(*cis*-3-fluorocyclobutyl)thiourea and 1-Cyclohexyl-3-(*trans*-3-fluorocyclobutyl)thiourea

Chemical formula: C₁₁H₁₉FN₂S

Exact mass: 230.1253

Molecular weight: 230.35



Following the procedure for **91**, commercially available ammonium **95** (50.0 mg, 0.40 mmol, 1.0 equiv.), KHCO₃ (44 mg, 0.44 mmol, 1.1 equiv.), anhydrous MeCN (400 μ L) and commercially available CyNCS (56 μ L, 0.40 mmol, 1.0 equiv.) afforded 26.0 mg (0.11 mmol, 28%) of the title compound **96** and 43.8 mg (0.19 mmol, 48%) of the title compound **98** as amorphous white powders after purification by flash column chromatography.

96; *R_f* 0.30 (25% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3260 (m, N–H), 2932 (m, C–H), 3855 (m, Cy C–H), 1543 (m, N–H); δ_{H} (400 MHz, MeOH-*d*₄) 4.67 (1H, dddd, *J* = 56.1, 13.4, 6.6, 6.6 Hz, H-6), [4.09, 3.94] (2H, br s, H-4 and H-8), 2.74 (2H, dddd, *J* = 13.7, 6.6, 6.6, 3.1, 3.1 Hz, H-5 and H-7), 2.11 – 1.89 (2H, m, H-5 and H-7), 1.90 – 0.97 (10H, m, H-9, H-10, H-11, H-12 and H-13); δ_{C} (101 MHz, MeOH-*d*₄) 180.1 (s, C-2), 81.2 [d (d, *J* = 209.9 Hz), C-6], 52.5 (d, C-8), 38.69 (d, C-4), 38.65 [t (d, *J* = 19.9 Hz), C-5 and C-7], 32.3 (t, C-9 and C-13), 25.2 (t, C-11), 24.7 (t, C-10 and C-12); δ_{F} (376 MHz, MeOH-*d*₄) –169.7; *m/z* (ESI) (rel. int.) 233 (5), 232 (15), 231 (MH⁺, 100) [Found MH⁺, 231.1324. C₁₁H₁₉FN₂S requires MH⁺, 231.1331; Δ = –3.0 ppm].

98; *R_f* 0.20 (25% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3227 (m, N–H), 2931 (w, C–H), 2855 (w, Cy C–H), 1552 (m, N–H); δ_{H} (400 MHz, MeOH-*d*₄) 5.15 (1H, dddd, *J* = 56.5, 9.8, 6.6, 3.5 Hz, H-6), 4.77 (1H, br s, H-4), 4.07 (1H, br s, H-8), 2.59 (2H, dddd, *J* = 21.0, 14.3, 8.2, 3.5 Hz, H-5 and H-7), 2.33 (2H, ddd, *J* = 20.3, 20.2, 6.0 Hz, H-5 and H-7), 2.07 – 1.57 (5H, m, H-9, H-10, H-11, H-12 and H-13), 1.50 – 1.12 (5H, m, H-9, H-10, H-11, H-12 and H-13); δ_{C} (101 MHz, MeOH-*d*₄) 180.3 (s, C-2), 86.4 [d (d, *J* = 198.1 Hz) C-6], 52.6 (d, C-8), 44.6 (d, C-4), 37.6 [t (d, *J* = 22.3 Hz), C-5 and C-7], 32.3 (t, C-9 and C-13), 25.2 (t, C-11), 24.7 (t, C-10 and C-12); δ_{F} (376 MHz, MeOH-*d*₄) –178.7; *m/z* (ESI) (rel. int.) 277 (10), 276 (15), 275 (MCO₂H[–], 100) [Found MCO₂H[–], 275.1223. C₁₁H₁₉FN₂S requires MCO₂H[–], 275.1230; Δ = –2.5 ppm].

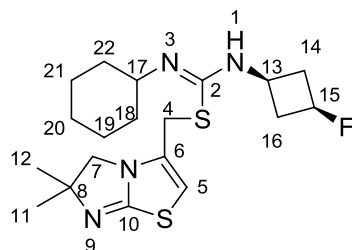
3.2.34. Compound **97**

Name: *N*'-Cyclohexyl-*N*-(*cis*-3-fluorocyclobutyl)[({6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl} methyl)sulfanyl]methanimidamide .xTFA

Chemical formula: C₁₉H₂₉FN₄S₂

Exact mass: 396.1818

Molecular weight of M.3TFA: 738.66



Following the procedure for **106**, chloride **52** (26.3 mg, 0.11 mmol, 1.0 equiv.), thiourea **96** (26.0 mg, 0.11 mmol, 1.0 equiv.) and EtOH (1.1 mL) afforded 51.7 mg (0.070 mmol, 64%) of the title compound as a white foam after purification by preparative HPLC, gradient 5–35% B over 20 min; $\nu_{\max}/\text{cm}^{-1}$ 2941 (w, C–H), 1670 (s, C=N), 1605 (m), 1560 (m), [1197, 1175, 1127] (s, C–F); δ_{H} (400 MHz, MeOH-*d*₄) 7.01 – 6.77 (1H, m, H-5), 4.85 (1H, dtt, *J* = 56.0, 6.6, 6.6 Hz, H-15), 4.67 – 4.43 (2H, m, H-4), 4.40 – 4.24 (2H, m, H-7), 4.22 – 3.62 (2H, m, H-13 and H-17), 3.09 – 2.78 (2H, m, H-14 and H-16), 2.75 – 2.20 (2H, m, H-14 and H-16), 2.03 – 1.68 (5H, m, H-18, H-19, H-20, H-21 and H-22), 1.67 – 1.56 (6H, s, H-11 and H-12), 1.55 – 1.10 (5H, m, H-18, H-19, H-20, H-21 and H-22); δ_{C} (101 MHz, MeOH-*d*₄) 171.1 (s, C-2), [164.6, 164.1] (s, C-10), 162.0 [s (q, *J* = 36.7, 35.6 Hz), CF₂CO₂H], [133.7, 133.5] (s, C-6), 118.0 [s (q, *J* = 289 Hz), CF₃CO₂H], 112.0 (d, C-5), 82.5 [d (d, *J* = 211.6 Hz), C-15], 82.3 [d (d, *J* = 211.3 Hz), C-15], 71.9 (s, C-8), [60.1, 60.0] (t, C-7), 59.2 (d, C-13), 56.3 (d, C-17), 43.7 [t (d, *J* = 25.3 Hz), C-14 and C-16], 42.0 [t (d, *J* = 26.1 Hz), C-14 and C-16], 40.3 [t (d, *J* = 21.7 Hz), C-14 and C-16], 39.6 [t (d, *J* = 23.2 Hz), C-14 and C-16], [34.3, 33.4] (t, C-18 and C-22), [30.0, 29.8] (t, C-4), 28.7 (q, C-11 and C-12), [26.6, 26.5, 26.4] (t, C-19, C-20 and C-21); δ_{F} (376 MHz, MeOH-*d*₄) –77.3 (TFA), –169.9 (sym. m.); *m/z* (ESI) (rel. int.) 397 (MH⁺, 15), 391 (20), 257 (35), 220 (100) [Found MH⁺, 397.1888. C₁₉H₂₉FN₄S₂ requires MH⁺, 397.1890; Δ = –0.6 ppm].

Experimental procedures

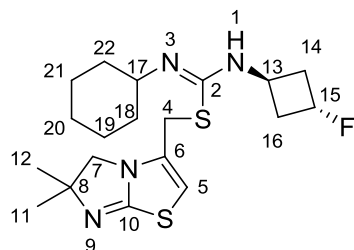
3.2.35. Compound **99**

Name: *N'*-Cyclohexyl-*N*-(*trans*-3-fluorocyclobutyl)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methanimidamide .xTFA

Chemical formula: C₁₉H₂₉FN₄S₂

Exact mass: 396.1818

Molecular weight of M.3TFA: 738.66



Following the procedure for **106**, chloride **52** (45.4 mg, 0.19 mmol, 1.0 equiv.), thiourea **96** (43.8 mg, 0.19 mmol, 1.0 equiv.) and EtOH (1.9 mL) afforded 66.2 mg (0.090 mmol, 47%) of the title compound as a white foam after purification by preparative HPLC, gradient 5–35% B over 20 min; $\nu_{\max}/\text{cm}^{-1}$ 2937 (w, C–H), 1670 (s, C=N), 1605 (m), 1560 (m), [1197, 1175, 1127] (s, C–F); δ_{H} (400 MHz, MeOH-*d*₄) 7.01 – 6.82 (1H, m, H-5), 5.38 – 5.09 (1H, m, H-15), 4.88 – 4.43 (3H, m, H4 and H-13), 4.32 (2H, s, H-7), 3.96 – 3.67 (1H, m, H-17), 2.83 – 2.43 (4H, m, H-14 and H-16), 2.04 – 1.69 (5H, m, H-18, H-19, H-20, H-21 and H-22), 1.62 (6H, s, H-11 and H-12), 1.55 – 1.10 (5H, m, H-18, H-19, H-20, H-21 and H-22); δ_{C} (101 MHz, MeOH-*d*₄) 171.1 (s, C-2), [164.7, 164.3] (s, C-10), 162.4 [s (q, *J* = 36.4, 35.4 Hz), CF₃CO₂H], [133.7, 133.6] (s, C-6), 118.2 [s (q, *J* = 290.0 Hz), CF₃CO₂H], 112.0 (d, C-5), 87.9 [d (d, *J* = 195.1 Hz), C-15], 87.6 [d (d, *J* = 200.0 Hz), C-15], 71.9 (s, C-8), 60.1 (t, C-7), [59.2, 56.2] (d, C-16), 47.6 [d (d, *J* = 5.9 Hz), C-13], [45.5, 42.7] (t, C-14 and C-16), 39.4 [t (d, *J* = 23.2 Hz), C-14 and C-16], 38.6 [t (d, *J* = 22.8 Hz), C-14 and C-16], [34.2, 33.4] (t, C-18 and C-22), [30.2, 30.04, 29.97] (t, C-4), 28.6 (q, C-11 and C-12), [26.7, 26.5, 26.4] (t, C-19, C-20 and C-21); δ_{F} (376 MHz, MeOH-*d*₄) –77.1 (TFA), –179.8 (sym. m); *m/z* (ESI) (rel. int.) 397 (MH⁺, 30), 315 (30), 257 (30), 222 (100) [Found MH⁺, 397.1900. C₁₉H₂₉FN₄S₂ requires MH⁺, 397.1890; Δ = 2.7 ppm].

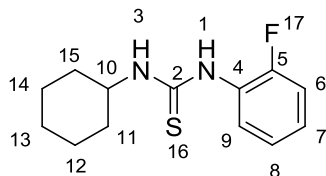
3.2.36. Compound **103**

Name: 3-Cyclohexyl-1-(2-fluorophenyl)thiourea

Chemical formula: $C_{13}H_{17}FN_2S$

Exact mass: 252.1096

Molecular weight: 252.35



Following the procedure for **104**, commercially available aniline **100** (1.00 g, 9.00 mmol, 1.0 equiv.), commercially available CyNCS (1.27 mg, 9.00 mmol, 1.0 equiv.) and EtOH (9 mL) afforded 1.68 g (6.68 mmol, 74%) of the title compound as a light pink amorphous solid after flash column chromatography; R_f 0.29 (25% ethyl acetate in petroleum ether); $\nu_{\max}/\text{cm}^{-1}$ 3239 (w, br, N–H), 3047 (w, ar C–H), 2932 (m, Cy C–H), 1536 (vs, N–H), 1338 (m, C=S), 1259 (m, C–F); δ_H (400 MHz, CDCl_3) 7.46 – 7.03 (4H, m, H-6, H-7, H-8 and H-9), 5.77 (2H, br s, H-1 and H-3), 4.18 (1H, s, H-10), 2.13 – 1.88 (2H, m, H-11 and H-15), 1.70 – 1.51 (3H, m, H-12, H-13 and H-14), 1.44 – 1.23 (2H, m, H-11 and H-15), 1.23 – 0.95 (3H, m, H-12, H-13 and H-14); δ_C (101 MHz, CDCl_3) 179.4 (s, C-2), 156.1 [s (d, $J = 249.6$ Hz), C-5], 128.4 [d (d, $J = 7.7$ Hz), C-7], 126.5 (d, C-8), 125.1 [d (d, $J = 3.9$ Hz), C-9], 117.1 [d (d, $J = 19.1$ Hz), C-6], 54.2 (d, C-10), 32.5 (t, C-11 and C-15), 25.4 (t, C-13), 24.7 (t, C-12 and C-14); δ_F (376 MHz, CDCl_3) –121.3; m/z (ESI) (rel. int.) 255 (5), 254 (15), 253 (MH^+ , 100), 241 (20) [Found MH^+ , 253.1168. $C_{13}H_{17}FN_2S$ requires MH^+ , 253.1175; $\Delta = -2.8$ ppm].

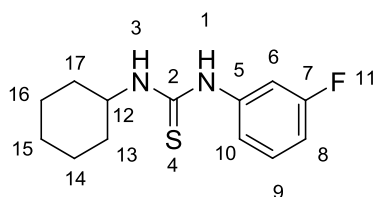
3.2.37. Compound **104**

Name: 3-(Cyclohexyl)-1-(3-fluorophenyl)thiourea

Chemical formula: $C_{13}H_{17}FN_2S$

Exact mass: 252.1096

Molecular weight: 252.35



The compound was synthesised following a modified literature procedure.¹⁸⁴ A pressure vial containing a solution of commercially available aniline **101** (192 μL , 2.0 mmol, 1.0 equiv.)

Experimental procedures

and commercially available CyNCS (283 mg, 2.0 mmol, 1.0 equiv.) in EtOH (2 mL) was sealed and the reaction mixture was allowed to stir at 90 °C overnight. The reaction mixture was then concentrated *in vacuo* and purified by flash column chromatography to afford 441 mg (1.75 mmol, 88%) of the title compound as a white amorphous powder; **R_f** 0.26 (25% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3228 (w, ar C–H and N–H), 2936 (m, C–H), 2853 (m, C–H), [1592, 1516, 1481] (m, ar C–C), 1211 (m, C–F and C=S); δ_{H} (400 MHz, MeOH-*d*₄) 7.40 (1H, dt, *J* = 10.9, 2.3 Hz, H-6), 7.32 (1H, td, *J* = 8.2, 6.5 Hz, H-9), 7.13 (1H, ddd, *J* = 8.2, 2.1, 1.0 Hz, H-10), 6.87 (1H, tdd, *J* = 8.5, 2.5, 0.9 Hz, H-8), 4.23 (1H, br s, H-12), 2.12 – 1.91 (2H, m, H-13 and H-17), 1.87 – 1.72 (2H, m, H-14 and H-16), 1.71 – 1.59 (1H, m, H-15), 1.52 – 1.34 (2H, m, H-13 and H-17), 1.34 – 1.12 (3H, m, H-14, H-15 and H-16); δ_{C} (101 MHz, CDCl₃) 179.1 (s, C-2), 131.5 [d (d, *J* = 9.1 Hz), C-9], 120.1 [d (d, *J* = 3.0 Hz), C-10], 114.0 [d (d, *J* = 21.2 Hz), C-8], 111.9 [d (d, *J* = 23.4 Hz), C-6], 54.3 (d, C-12), 32.5 (t, C-13 and C-17), 25.4 (t, C-15), 24.7 (t, C-14 and C-16); δ_{F} (376 MHz, MeOH-*d*₄) –114.3; **m/z** (ESI) (rel. int.) 255 (5), 254 (15), 253 (MH⁺, 100) [Found MH⁺, 253.1175. C₁₃H₁₇FN₂S requires MH⁺, 253.1175; Δ = 0.0 ppm].

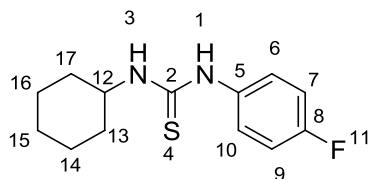
3.2.38. Compound **105**

Name: 3-(Cyclohexyl)-1-(4-fluorophenyl)thiourea

Chemical formula: C₁₃H₁₇FN₂S

Exact mass: 252.1096

Molecular weight: 252.35



Following the procedure for **104**, commercially available aniline **102** (192 μ L, 2.0 mmol, 1.0 equiv.), commercially available CyNCS (283 mg, 2.0 mmol, 1.0 equiv.) and EtOH (2 mL) afforded 358 mg (1.42 mmol, 71%) of the title compound as an off-white amorphous solid after flash column chromatography; **R_f** 0.55 (50% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3380 (w, N–H), 3137 (w, ar C–H), 2936 (m, C–H), [1533, 1507] (m, ar C–C), 1210 (st, C–F and/or C=S); δ_{H} (400 MHz, CDCl₃) 7.64 (1H, br s, H-1), 7.23 – 7.06 (4H, m, H-6, H-7, H-9 and H-10), 5.68 (1H, br s, H-3), 4.23 (1H, m, H-12), 2.14 – 1.95 (2H, m, H-13 and H-17), 1.68 – 1.56 (3H, m, H-14, H-15 and H-16), 1.45 – 1.32 (2H, m, H-14 and H-16), 1.19 – 1.02 (3H, m, H-13, H-15 and H-17); δ_{C} (101 MHz, CDCl₃) 179.8 (s, C-2), 161.6 [s (d,

$J = 248.4$ Hz), C-8], 132.1 (s, C-5), 127.8 [d (d, $J = 8.6$ Hz), C-6 and C-10], 117.3 [d (d, $J = 22.7$ Hz), C-7 and C-9], 54.2 (d, C-12), 32.7 (t, C-13 and C-17), 25.5 (t, C-15), 24.8 (t, C-14 and C-16); δ_F (376 MHz, MeOH- d_4) -119.0 ; m/z (ESI) (rel. int.) 569 (20), 568 (10), 567 (M_2Cu^+ , 35), 254, (15), 253 (MH^+ , 100) [Found MH^+ , 253.1169. $C_{13}H_{17}FN_2S$ requires MH^+ , 253.1175; $\Delta = -2.4$ ppm].

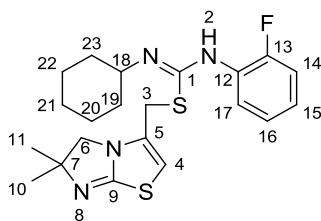
3.2.39. Compound **106**

Name: *N'*-Cyclohexyl-*N*-(2-fluorophenyl)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methanimidamide .xTFA

Chemical formula: $C_{21}H_{27}FN_4S_2$

Exact mass: 418.1661

Molecular weight of M.3TFA: 760.66



A mixture of chloride **52** (120 mg, 0.5 mmol, 1.0 equiv.) and thiourea **105** (126 mg, 0.50 mmol, 1.0 equiv.) in anhydrous MeCN (5 mL) and EtOH (300 μ L) was allowed to stir at 35 °C for 7 days. The reaction mixture was concentrated *in vacuo* and taken up in H_2O + 0.1% TFA. Purification of this solution by preparative HPLC, gradient 15–25% B over 20 min, afforded 118 mg (0.16 mmol, 31%) of the title compound as a white amorphous powder; δ_H (400 MHz, MeOH- d_4) 7.75 – 7.29 (4H, m, H-14, H-15, H-16 and H-17), 4.64 – 3.67 (4H, m, H-3 and H-6), 3.35 (1H, br s, H-18), 2.03 – 1.57 (5H, m, H-19, H-20, H-21, H-22 and H-23), 1.57 – 1.38 (6H, m, H-10 and H-11), 1.38 – 0.92 (5H, m, H-19, H-20, H-21, H-22 and H-23); δ_C (101 MHz, MeOH- d_4) 171.8 (s, C-1), 170.3 (s, C-9), 161.2 [s (d, $J = 34.8$ Hz), C-12], 159.3 [s (d, $J = 249.5$ Hz), C-13], 134.6 [d (d, $J = 8.8$ Hz), C-15], 131.6 (d, C-16), 130.4 (d, C-4), 127.2 [d (d, $J = 3.5$ Hz), C-17], 117.7 [d (d, $J = 20.0$ Hz), C-14], 89.0 (s, C-5), 70.9 (s, C-7), 61.1 (d, C-18), 55.8 (t, C-6), 35.8 (t, C-3), [31.4, 31.3] (t, C-19 and C-23), [27.1, 27.0, 26.9, 26.43, 26.39] (q, C-10 and C-11), 24.5 (t, C-20 and C-22), 24.4 (t, C-21); δ_F (376 MHz, MeOH- d_4) -76.9 (TFA), -122.1 (ddd, $J = 12.1, 6.6, 6.6$ Hz, ar F); m/z (ESI) (rel. int.) 421 (10), 420 (25), 419 (MH^+ , 100), 337 (25), 297 (35) [Found MH^+ , 419.1725. $C_{21}H_{27}FN_4S_2$ requires MH^+ , 419.1739; $\Delta = -3.3$ ppm].

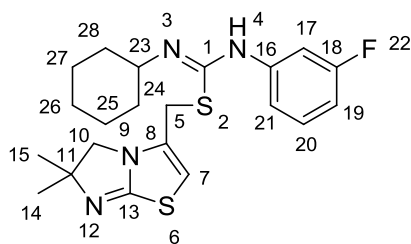
3.2.40. Compound **107**

Name: *N*'-Cyclohexyl-*N*-(3-fluorophenyl)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methanimidamide .xHCl

Chemical formula: C₂₁H₂₇FN₄S₂

Exact mass: 418.1661

Molecular weight of M.2HCl: 491.52



A mixture of chloride **52** (120 mg, 0.5 mmol, 1.0 equiv.) and thiourea **104** (126 mg, 0.50 mmol, 1.0 equiv.) in anhydrous MeCN (5 mL) and EtOH (100 μ L) was allowed to stir at 35 °C for 7 days. Having formed a precipitate, the reaction mixture was centrifuged (5 min, 5 krpm, 4 °C) and the supernatant was discarded. The pellet was resuspended in anhydrous MeCN (1 mL), the slurry was centrifuged as aforementioned and the supernatant was discarded. The pellet was resuspended in Et₂O (50 mL), the slurry was centrifuged as aforementioned and the supernatant was discarded. The pellet was dried under high vacuum to afford 149 mg (0.30 mmol, 61%) of the title compound as a white amorphous powder; **R_f** 0.54 (50% methanol in ethyl acetate); $\nu_{\text{max}}/\text{cm}^{-1}$ 3423 (w, br, N⁺-H), 2947 (w, C-H), 1588 (m, ar C-C); δ_{H} (400 MHz, DMSO-*d*₆) 10.48 (1H, br s, H-12), 10.19 – 9.22 (1H, br s, H-4), 7.54 – 7.38 (1H, m, H-21), 7.29 – 7.11 (2H, m, H-17 and H-20), 7.11 – 6.99 (1H, m, H-19), 6.87 (1H, s, H-7), 4.66 (2H, s, H-5), 3.98 (2H, s, H-10), 1.95 – 1.83 (2H, m, H-24 and H-28), 1.78 – 1.66 (3H, m, H-25, H-26 and H-27), 1.45 (6H, s, H-14 and H-15), 1.36 – 1.20 (3H, m, H-24, H-26 and H-28), 1.17 – 1.05 (1H, m, H-25 and H-27); δ_{C} (101 MHz, D₂O) 167.6 (s, C-1), 161.5 [s (d, *J* = 250 Hz), C-18], 161.1 (s, C-13), 135.8 (s, C-16), 130.7 (d, C-21), 130.5 (s, C-8), 121.7 (d C-20), 115.3 [d (d, *J* = 20.6 Hz), C-17], 113.0 [d (d, *J* = 23.7 Hz), C-19], 108.4 (d, C-7), 69.1 (s, C-11), 56.8 (t, C-10), 54.3 (d, C-23), 30.2 (t, C-24 and C-28), 28.6 (t, C-5), 26.0 (q, C-14 and C-15), 23.5 (t, C-26), 23.3 (t, C-25 and C-27); δ_{F} (376 MHz, MeOH-*d*₄) – 112.0; **m/z** (ESI) (rel. int.) 421 (10), 420 (25), 419 (MH⁺, 98), 337 (100) [Found MH⁺, 419.1739. C₂₁H₂₇FN₄S₂ requires MH⁺, 419.1739; Δ = 0.0 ppm].

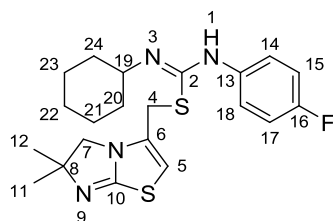
3.2.41. Compound **108**

Name: *N'*-Cyclohexyl-*N*-(4-fluorophenyl)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methanimidamide .xHCl

Chemical formula: C₂₁H₂₇FN₄S₂

Exact mass: 418.1661

Molecular weight of M.2HCl: 491.52



Following the procedure for **107**, chloride **52** (120 mg, 0.5 mmol, 1.0 equiv.), thiourea **105** (126 mg, 0.50 mmol, 1.0 equiv.) and anhydrous MeCN (5 mL) and EtOH (200 μ L) afforded 102 mg (0.21 mmol, 42%) of the title compound as a white amorphous powder; $\nu_{\text{max}}/\text{cm}^{-1}$ 3376 (w, br, N⁺-H), 2931 (w, C-H), 1589 (m, ar C-C); δ_{H} (400 MHz, MeOH-*d*₄) 7.44 (2H, br s, H-14 and H-18), 7.28 (2H, dd, *J* = 8.6, 8.6 Hz, H-15 and H-17), 6.89 (1H, s, H-5), 4.43 (2H, br s, H-4), 4.06 (2H, br s, H-7), 3.85 (1H, s, H-19), 2.18 – 1.66 (5H, m, H-20, H-21, H-22, H-23 and H-24), 1.55 (6H, s, H-11 and H-12), 1.50 – 1.08 (3H, m, H-21, H-22 and H-23); δ_{C} (101 MHz, MeOH-*d*₄) 169.0 (s, C-2), 131.6 (s, C-13), 129.2 (d, C-14 and C-18), 116.4 [d (d, *J* = 19.6 Hz), C-15 and C-17], 109.9 (d, C-5), 85.7 (s, C-6), 69.8 (s, C-8), 58.0 (t, C-7), 55.4 (d, C-19), 31.4 (t, C-20 and C-24), 28.4 (t, C-4), 26.6 (q, C-11 and C-12), 24.5 (t, C-21, C-22 and C-23); δ_{F} (376 MHz, MeOH-*d*₄) -113.4; *m/z* (ESI) (rel. int.) 421 (10), 420 (25), 419 (MH⁺, 98), 337 (10) [Found MH⁺, 419.1738. C₂₁H₂₇FN₄S₂ requires MH⁺, 419.1739; Δ = -0.2 ppm].

Experimental procedures

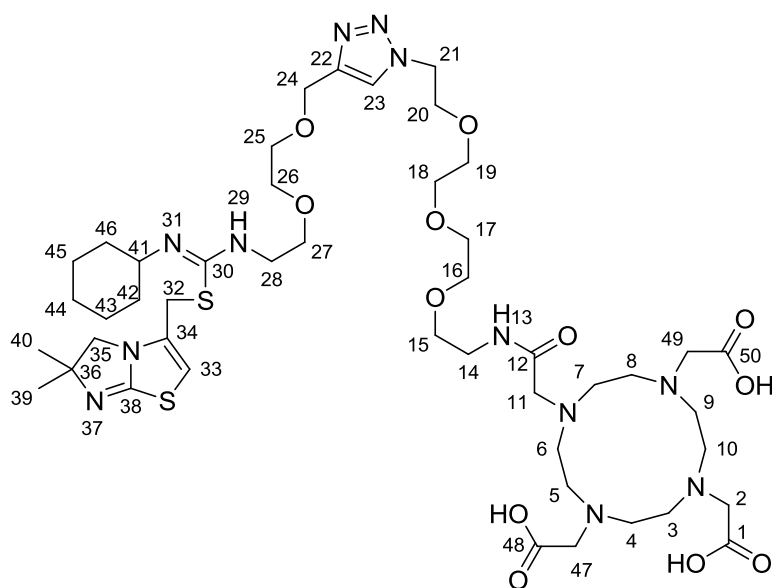
3.2.42. Compound **110**

Name: 2-[4,10-bis(Carboxymethyl)-7-[(2-[2-(2-[2-[4-(*N*-cyclohexyl)-1-{2-[2-({[(6,6-dimethyl-5*H*,6*H*-imidazo{2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanylmethanimidoyl}amino)ethoxy]ethoxy)methyl)-1*H*-1,2,3-triazol-1-yl]ethoxy)ethoxy)ethoxy]ethyl}carbamoyl)methyl]-1,4,7,10-tetraazacyclododecan-1-yl]acetic acid .xTFA

Chemical formula: C₄₆H₇₈N₁₂O₁₂S₂

Exact mass: 1054.5304

Molecular weight of M.7TFA: 1853.48

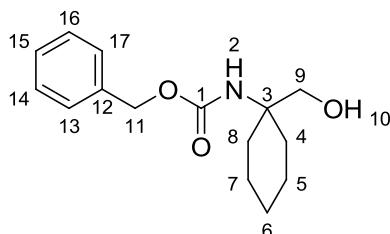


tert-Butyl ester **133** (5.73 mg, 3.00 μmol) was dissolved a mixture of TFA–CH₂Cl₂ (1:1) (71 μL) and the reaction mixture was allowed to stir at r.t. overnight before concentrating *in vacuo*. The crude residue was dissolved in H₂O + 0.1% TFA (4 mL) and purified by preparative HPLC, gradient 5–50% B over 20 min, to afford 1.66 mg (0.95 μmol, 32%) of the title compound as a colourless glass; δ_H (400 MHz, MeOH-*d*₄) 8.02 – 7.91 (1H, m, H-23), [4.58 – 4.40, 4.05 – 3.27, 3.15 – 2.63] (4H+39H+12H, 3×m, H-2, H-3, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-14, H-15, H-16, H-17, H-18, H-19, H-20, H-21, H-24, H-25, H-26, H-27, H-28, H-32, H-35, H-41, H-47 and H-49), 2.25 – 1.50 (5H, m, H-42, H-43, H-44, H-45 and H-46), 1.49 – 1.34 (6H, m, H-39 and H-40), 1.33 – 1.13 (5H, m, H-42, H-43, H-44, H-45 and H-46); δ_F (376 MHz, MeOH-*d*₄) –77.0 (TFA); *m/z* (ESI) (rel. int.) 528 (MH₂²⁺, 100) [Found MH₂²⁺, 528.2715. C₄₆H₇₈N₁₂O₁₂S₂ requires MH₂²⁺, 528.2725; Δ = –1.9 ppm].

3.2.43. Compound **115**¹⁸⁹Name: Benzyl *N*-[1-(hydroxymethyl)cyclohexyl]carbamateChemical formula: C₁₅H₂₁NO₃

Exact mass: 263.1521

Molecular weight: 263.33



The compound was synthesised adapting a literature procedure.¹⁹⁰ To a solution of CbzCl (355 μ L, 3.95 mmol, 1.0 equiv.) in anhydrous CH₂Cl₂ (4 mL) chilled to 0 °C and under an atmosphere of N₂ was added a solution of commercially available amine **117** (1.018 g, 7.89 mmol, 2.0 equiv.) in anhydrous CH₂Cl₂ (8 mL) dropwise over 20 min. The reaction mixture was allowed to stir at 0 °C for 10 min and then at r.t. for 3.5 h and filtered. The cake was washed with CH₂Cl₂ (20 mL) and the filtrate concentrated *in vacuo*. The crude residue was taken up in ethyl acetate – petroleum ether (1:1), filtered through a plug of silica and washed with ethyl acetate – petroleum ether (1:1) (150 mL). The filtrate was concentrated *in vacuo* to afford 609 mg (2.32 mmol, 59%) of the title compound as a white amorphous powder; *R_f* 0.33 (50% ethyl acetate in petroleum ether); $\nu_{\text{max}}/\text{cm}^{-1}$ 3425 (m, N–H st), 3284 (m, O–H), 2931 (s), 1684 (vs, C=O), 1534 (m, N–H δ), 1258 (s), 1240 (s); δ_{H} (400 MHz, CDCl₃) 7.42 – 7.29 (5H, m, H-13, H-14, H-15, H-16 and H-17), 5.07 (2H, s, H-9), 4.78 (1H, br s, H-2), 3.70 (2H, s, H-11), 1.87 – 1.77 (2H, m, H-4 and H-8), 1.60 – 1.28 (9H, m, H-4, H-5, H-6, H-7, H-8 and H-10); δ_{C} (101 MHz, CDCl₃) 156.2 (s, C-1), 136.3 (s, C-12), 128.6 (d, C-14 and C-16), 128.3 (d, C-15), 128.1 (d, C-13 and C-17), 69.6 (t, C-11), 66.8 (t, C-9), 56.6 (s, C-3), 32.2 (t, C-4 and C-8), 25.6 (t, C-6), 21.4 (t, C-5 and C-7); *m/z* (ESI) (rel. int.) 286 (MNa⁺, 30), 265 (20), 264 (MH⁺, 85), 220 (100) [Found MH⁺, 264.1599. C₁₅H₂₁NO₃ requires MH⁺, 264.1600; Δ = –0.4 ppm].

Experimental procedures

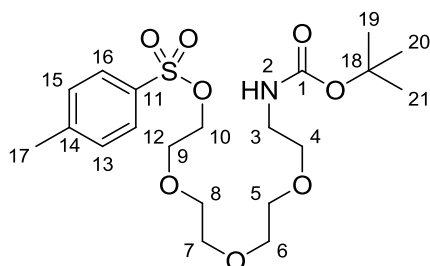
3.2.44. Compound **116**^{191,192}

Name: *tert*-Butyl *N*-{2-[2-(2-{2-[(4-methylbenzenesulfonyl)oxy]ethoxy}ethoxy)ethoxy]ethyl} carbamate

Chemical formula: C₂₀H₃₃NO₈S

Exact mass: 447.1927

Molecular weight: 447.54

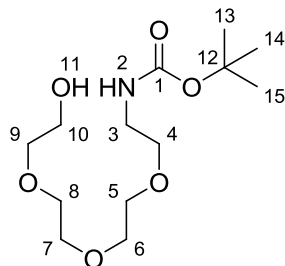


The compound was synthesised following a modified literature procedure.¹⁹¹ To a solution of alcohol **125** (759 mg, 2.59 mmol, 1.0 equiv.) in CH₂Cl₂ (26 mL) at 0 °C were added NEt₃ (831 µL, 5.96 mmol, 2.3 equiv.) and TsCl (543 mg, 2.85 mmol, 1.1 equiv.) sequentially. The reaction mixture was allowed to stir at 0 °C for 5 min and at r.t. over 3 days before diluting with CH₂Cl₂ (150 mL) and washing with brine (1×50 mL). The organic layer was dried over MgSO₄ and concentrated *in vacuo*. The crude residue was purified by flash column chromatography to afford 377.8 mg (0.85 mmol, 33%) of the title compound as a colourless oil; *R*_f 0.32 (75% ethyl acetate in petroleum ether); *v*_{max}/cm⁻¹ 3360 (w, br, N–H), 2871 (w), 1708 (s, C=O), 1362 (s, O=S=O st as), 1175 (vs, O=S=O st sym), 1096 (s), 920 (s), 663 (s); *δ*_H (400 MHz, CDCl₃) 7.70 (2H, d, *J* = 8.3 Hz, H-12 and H-16), 7.26 (2H, d, *J* = 8.1 Hz, H-13 and H-15), 5.09 (1H, s, H-2), 4.09 – 4.04 (2H, m, H-10), 3.62 – 3.57 (2H, m, H-9), 3.54 – 3.46 (8H, m, H-5, H-6, H-7 and H-8), 3.43 (2H, t, *J* = 5.3 Hz, H-4), 3.20 (2H, dt, *J* = 5.2, 5.2 Hz, H-3), 2.35 (2H, s, H-17), 1.34 (9H, s, H-19, H-20 and H-21); *δ*_C (101 MHz, CDCl₃) 155.9 (s, C-1), 144.8 (s, C-14), 132.9 (s, C-11), 129.8 (d, C-13 and C-15), 127.9 (d, C-12 and C-16), 78.9 (s, C-18), [70.6, 70.4, 70.4, 70.1, 70.0, 69.3, 68.5] (t, C-4, C-5, C-6, C-7, C-8, C-9 and C-10), 40.2 (t, C-3), 28.3 (q, C-19, C-20 and C-21), 21.5 (q, C-17); *m/z* (ESI) (rel. int.) 472 (5), 471 (20), 470 (MNa⁺, 100), 348 (10) [Found MNa⁺, 470.1808. C₂₀H₃₃NO₈S requires MNa⁺, 470.1825; Δ = –3.6 ppm].

3.2.45. Compound **133**^{185,186}Name: *tert*-Butyl *N*-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)carbamate^{193,194}Chemical formula: C₁₃H₂₇NO₆

Exact mass: 293.1838

Molecular weight: 293.36



The compound was synthesised following a modified literature procedure.¹⁹¹ A solution of commercially available amine **124** (1.00 g, 5.18 mmol, 1.0 equiv.) in CH₂Cl₂ (20 mL) chilled to 0 °C was treated with a solution of Boc₂O (1.24 g, 5.70 mmol, 1.1 equiv.) in CH₂Cl₂ (17 mL). The reaction mixture was allowed to stir at 0 °C for 5 min and then at r.t. overnight before diluting with CH₂Cl₂ (50 mL) and washing with brine (1×20 mL). The aqueous layer was extracted with CH₂Cl₂ (3×50 mL) and the combined organic layers were dried over Na₂SO₄ and concentrated *in vacuo*. The crude residue was purified by flash column chromatography to afford 948.2 mg (3.24 mmol, 62%) of the title compound as a fluent light yellow oil; *R_f* 0.14 (75% ethyl acetate in petroleum ether); *v*_{max}/cm⁻¹ 3352 (w, br, N–H and O–H), 2870 (m), 1692 (s, C=O), 1522 (m), 1365 (m), 1249 (m), 1099 (vs); *δ*_H (400 MHz, CDCl₃) 5.67 (1H, br s, H-2), 3.78 – 3.69 (2H, m, H-10), 3.72 – 3.59 (10H, m, H-5, H-6, H-7, H-8 and H-9), 3.54 (2H, t, *J* = 5.0 Hz, H-4), 3.33 (2H, dt, *J* = 5.0, 4.9 Hz, H-3), 3.13 (1H, br s, H-11), 1.45 (9H, s, H-13, H-14 and H-15); *δ*_C (101 MHz, CDCl₃) 156.2 (s, C-1), 79.0 (s, C-12), [72.6, 70.6, 70.5, 70.4, 70.3, 70.1] (t, C-4, C-5, C-6, C-7, C-8 and C-9), 61.7 (t, C-10), 40.4 (t, C-3), 28.4 (q, C-13, C-14 and C-15); *m/z* (ESI) (rel. int.) 317 (15), 316 (MNa⁺, 100), 305 (20), 260 (20), 208 (40) [Found MNa⁺, 316.1745. C₁₃H₂₇NO₆ requires MNa⁺, 316.1736; Δ = 2.8 ppm].

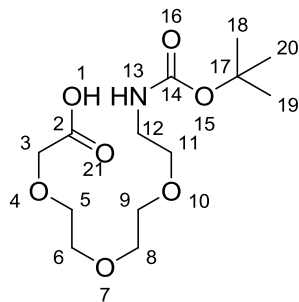
3.2.46. Compound **126**¹⁹⁵

Name: 2-{2-[2-(2-{[(*tert*-Butoxy)carbonyl]amino}ethoxy)ethoxy]ethoxy}acetic acid

Chemical formula: C₁₃H₂₅NO₇

Exact mass: 307.1631

Molecular weight: 307.34



The compound was synthesised adapting a literature procedure.¹⁷⁰ To alcohol **125** (948.2 mg, 3.24 mmol, 1.0 equiv.) was added a solution of TEMPO (35.9 mg, 0.23 mmol, 7 mol%) in MeCN (15.7 mL), a solution of NaClO₂ (80%, 713 mg, 6.48 mmol, 2.0 equiv.) in water (3.18 mL) and phosphate buffer (11.8 mL, 0.67 M, pH 6.7). The reaction mixture was allowed to stir for 10 min at r.t., treated with a solution of NaClO (1.58 mL, 0.28% w/v, 2 mol%) dropwise and allowed to stir at 35 °C overnight. The mixture was then quenched with 0.1 N NaOH_(aq) to pH 8 (approximately 4 mL), treated with a solution of Na₂SO₃ (986 mg, 7.83 mmol, 2.4 equiv.) in water (16 mL) dropwise and finally allowed to stir for 5 min. CH₂Cl₂ (20 mL) was then added and the heterogenous mixture was allowed to vigorously stir for 30 min. The organic layer was discarded and the aqueous one was slowly acidified with 2 N HCl_(aq) to pH 3 and extracted with CH₂Cl₂ (7×20 mL, monitored by charring with bromocresol green on silica plate). The combined organic layers were dried over Na₂SO₄ and concentrated *in vacuo* to afford 761.4 mg (2.48 mmol, 77%) of the title compound as a colourless oil; *R*_f 0.00–0.20 (ethyl acetate); *v*_{max}/cm⁻¹ 3345 (w, br, COO–H and N–H), 2873 (m), 1708 (s, C=O), 1518 (m), 1103 (vs); *δ*_H (400 MHz, CDCl₃) 6.80 (1H, br s, H-1), 5.13 (1H, br s, H-13), 4.16 (2H, s, H-3), 3.83 – 3.57 (8H, m, H-5, H-6, H-8 and H-9), 3.57 – 3.47 (2H, t, *J* = 5.18 Hz, H-11), 3.34 – 3.23 (2H, m, H-12), 1.43 (9H, s, H-18, H-19 and H-20); *δ*_C (101 MHz, CDCl₃) 172.5 (s, C-2), 156.2 (s, C-15), 79.4 (s, C-17), [71.3, 70.5, 70.3, 70.3, 70.0] (t, C-5, C-6, C-8, C-9 and C-11), 68.8 (t, C-3), 40.3 (t, C-12), 28.4 (q, C-18, C-19 and C-20); *m/z* (ESI) (rel. int.) 331 (15), 330 (MNa⁺, 100), 274 (MNa-*t*Bu⁺, 10), 208 (MH-Boc⁺, 10) [Found MNa⁺, 330.1516. C₁₃H₂₅NO₇ requires MNa⁺, 330.1529; Δ = –3.9 ppm].

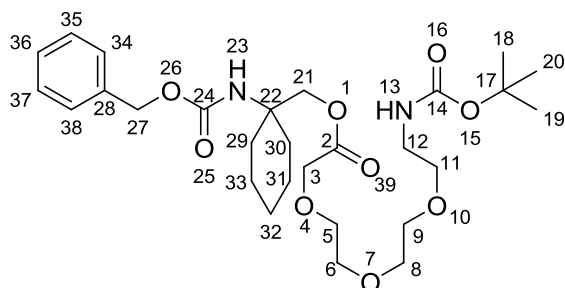
3.2.47. Compound **127**

Name: (1-{[(Benzyloxy)carbonyl]amino}cyclohexyl)methyl 2-{2-[2-(2-{[(*tert*-butoxy)carbonyl]amino}ethoxy)ethoxy]ethoxy}acetate

Chemical formula: C₂₈H₄₄N₂O₉

Exact mass: 552.3047

Molecular weight: 552.66



The compound was synthesised following a modified literature procedure.¹⁹⁶ A mixture of alcohol **115** (609 mg, 2.32 mmol, 1.2 equiv.), carboxylic acid **126** (594 mg, 1.93 mmol, 1.0 equiv.) and 4-dimethylaminopyridine (DMAP) (1.0 mg, 8.2 μmol, 0.4%mol) in anhydrous CH₂Cl₂ (23 mL) under an atmosphere of N₂ and chilled at 0 °C was treated with EDCI.HCl (407 mg, 2.13 mmol, 1.1 equiv.). The reaction mixture was allowed to stir at 0 °C for 5 min and at r.t. overnight, diluted with CH₂Cl₂ (90 mL) and washed with H₂O (35 mL) and brine (35 mL). The organic layer was dried over Na₂SO₄ and concentrated *in vacuo*. The crude residue was purified by flash column chromatography to afford 944.9 mg (1.71 mmol, 89%) of the title compound as a colourless oil; *R_f* 0.33 (50% ethyl acetate in petroleum ether); *v*_{max}/cm⁻¹ 3425 (m, N–H st), 3284 (m, O–H), 2931 (s), 1684 (vs, C=O), 1534 (m, N–H δ), 1258 (s), 1240 (s); *δ*_H (400 MHz, CDCl₃) 7.42 – 7.29 (5H, m, H-13, H-14, H-15, H-16 and H-17), 5.07 (2H, s, H-9), 4.78 (1H, br s, H-2), 3.70 (2H, s, H-11), 1.87 – 1.77 (2H, m, H-4 and H-8), 1.60 – 1.28 (9H, m, H-4, H-5, H-6, H-7, H-8 and H-10); *δ*_C (101 MHz, CDCl₃) 156.2 (s, C-1), 136.3 (s, C-12), 128.6 (d, C-14 and C-16), 128.3 (d, C-15), 128.1 (d, C-13 and C-17), 69.6 (t, C-11), 66.8 (t, C-9), 56.6 (s, C-3), 32.2 (t, C-4 and C-8), 25.6 (t, C-6), 21.4 (t, C-5 and C-7); *m/z* (ESI) (rel. int.) 286 (MNa⁺, 30), 265 (20), 264 (MH⁺, 85), 220 (100) [Found MH⁺, 264.1599. C₁₅H₂₁NO₃ requires MH⁺, 264.1600; Δ = –0.4 ppm].

Experimental procedures

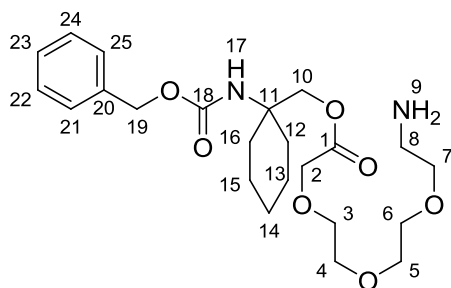
3.2.48. Compound **128**

Name: (1-[[{(Benzyloxy)carbonyl]amino}cyclohexyl)methyl 2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}acetate .xTFA

Chemical formula: C₂₃H₃₆N₂O₇

Exact mass: 452.2523

Molecular weight of M.TFA: 566.56



A solution of carbamate **127** (944.9 mg, 1.71 mmol, 1.0 equiv.) in CH₂Cl₂ (8 mL) chilled at 0 °C was slowly treated with TFA (8 mL) and the reaction mixture was allowed to stir at r.t. for 15 min before quenching with saturated Na₂CO_{3(aq)} (10 mL). The aqueous layer was extracted with CH₂Cl₂ (3×50 mL) and the combined organic layers were dried over Na₂SO₄ and concentrated *in vacuo* to afford 682.0 mg (1.51 mmol, 88%) of the title compound as a colourless oil; **R_f** 0.00–0.14 (ethyl acetate); **v_{max}**/cm⁻¹ 2919 (m, Cy C–H), 1677 (s, C=O), 1513 (m, N–H), 1138 (vs, CH₂–O–CH₂); **δ_H** (400 MHz, CDCl₃) 9.24 (1H, br s, CF₃CO₂H), 7.72 (3H, br s, H-9), 7.44 – 7.31 (5H, m, H-21, H-22, H-23, H-24 and H-25), 5.08 (2H, s, H-19), 4.75 (1H, br s, H-17), 4.35 (2H, s, H-2), 4.16 (2H, s, H-10), 3.89 – 3.79 (2H, m, H-7), 3.78 – 3.62 (8H, m, H-3, H-4, H-5 and H-6), 3.29 – 3.15 (2H, m, H-8), 2.08 – 1.94 (2H m, H-12 and H-16), 1.69 – 1.21 (8H, m, H-12, H-13, H-14, H-15 and H-16); **δ_C** (101 MHz, CDCl₃) 171.6 (s, C-1), 160.99 [s (d, *J* = 37.3 Hz), C-26], 154.6 (s, C-18), 136.5 (s, C-20), 128.6 (d, C-22 and C-24), 128.2 (d, C-23), 128.0 (d, C-21 and C-25), [70.2, 70.1, 70.0, 69.7] (t, C-3, C-4, C-5 and C-6), 68.7 (t, C-2), 68.0 (t, C-10), 66.9 (t, C-7), 66.3 (t, C-19), 54.5 (s, C-11), 39.9 (t, C-8), 31.7 (t, C-12 and C-16), 25.4 (t, C-14), 20.9 (t, C-13 and C-15); **δ_F** (376 MHz, CDCl₃) –75.85 (TFA); **m/z** (ESI) (rel. int.) 515 (70), 493 (100), 475 (MNa⁺, 20), 454 (10), 453 (MH⁺, 60) [Found MH⁺, 453.2594. C₂₃H₃₆N₂O₇ requires MH⁺, 453.2601; Δ = –1.5 ppm].

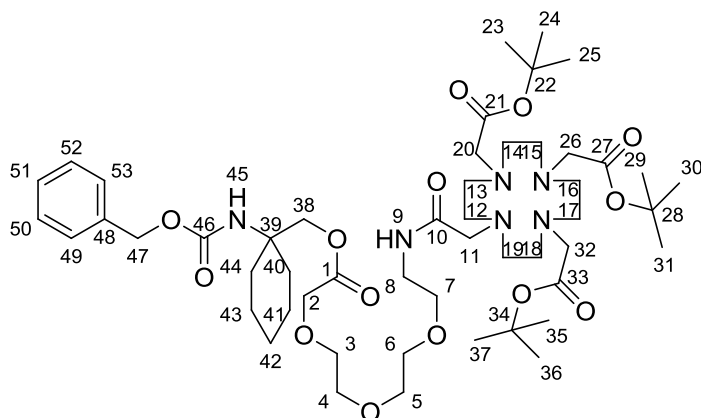
3.2.49. Compound **129**

Name: (1-{[(Benzyloxy)carbonyl]amino}cyclohexyl)methyl 2-(2-{2-[2-(2-{4,7,10-*tris*[2-(*tert*-butoxy)-2-oxoethyl]-1,4,7,10-tetraazacyclododecane-1-yl}acetamido)ethoxy]ethoxy}ethoxy)acetate

Chemical formula: C₅₁H₈₆N₆O₁₄

Exact mass: 1006.6202

Molecular weight: 1007.26



To a solution of carboxylic acid **113** (69.9 mg, 0.122 mmol, 1.1 equiv.), HOBt (19.5 mg, 0.144 mmol, 1.3 equiv.), HBTU (50.4 mg, 0.133 mmol, 1.2 equiv.) and DIPEA (50 μ L) in CH₂Cl₂ (500 μ L) was added a solution of amine **128** (50.0 mg, 0.111 mmol, 1.0 equiv.) in CH₂Cl₂ (500 μ L) and the reaction mixture was allowed to stir at r.t. overnight before concentrating *in vacuo*. The crude residue was taken up in CHCl₃ (10 mL), washed with water (3 $\times$ 10 mL) and brine (1 $\times$ 10 mL) and the organic layer was concentrated *in vacuo*. The residue was taken up in EtOAc (10 mL), washed with water (3 $\times$ 10 mL) and brine (1 $\times$ 10 mL), dried over Na₂SO₄ and concentrated *in vacuo* before being purified by flash column chromatography to afford 77.2 mg (0.077 mmol, 69%) of the title compound as a colourless oil; *R*_f 0.09 (1% methanol in methylene chloride); ν_{max} /cm⁻¹ 3406 (w, br, N-H), 2935 (w), 1708 (s, C=O), 1365 (m), 1223 (s), 840 (vs); δ_{H} (400 MHz, CDCl₃) 7.37 – 7.30 (5H, m, H-49, H-50, H-51, H-52 and H-53), 5.11 – 1.50 (54H, m, H-2, H-3, H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-13, H-14, H-15, H-16, H-17, H-18, H-19, H-20, H-26, H-32, H-38, H-40, H-41, H-42, H-43, H-44, H-45 and H-47), 1.49 – 1.37 (27H, m, H-23, H-24, H-25, H-39, H-30, H-31, H-35, H-36 and H-37); δ_{C} (101 MHz, CDCl₃) 173.7 (s, C-10), 173.1 (s, C-21, C-27, C-33), 173.0 (s, C-1), 156.4 (s, C-46), [136.6, 136.4] (s, C-48), [128.71, 128.68] (d, C-50 and C-52), [128.4, 128.3] (d, C-51), [128.2, 127.9] (d, C-49 and C-53), 82.13 (s, C-22 and C-34), 82.09 (s, C-28), 69.7 (t, C-20, C-26 and C-32), 68.3 (t, C-38), 66.9 (t, C-2), 66.3 (t, C-47),

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[56.7, 55.8, 55.7, 55.0, 54.5] (t, C-3, C-4, C-5, C-6 and C-7), 53.5 – 47.0 (t, C-12, C-13, C-14, C-15, C-16, C-17, C-18 and C-19), 38.7 (t, C-8), 32.3 (t, C-40 and C-44), 28.04 (q, C-23, C-24, C-25, C-35, C-36 and C-37), 28.01 (q, C-29, C-30 and C-31), [25.7, 25.5] (t, C-42), [21.5, 21.1] (t, C-41 and C-43); *m/z* (ESI) (rel. int.) 1030 (MNa⁺, 5), 1009 (6), 1008 (MH⁺, 9) [Found MH⁺, 1007.6300. C₅₁H₈₆N₆O₁₄ requires MH⁺, 1007.6280; Δ = 2.0 ppm].

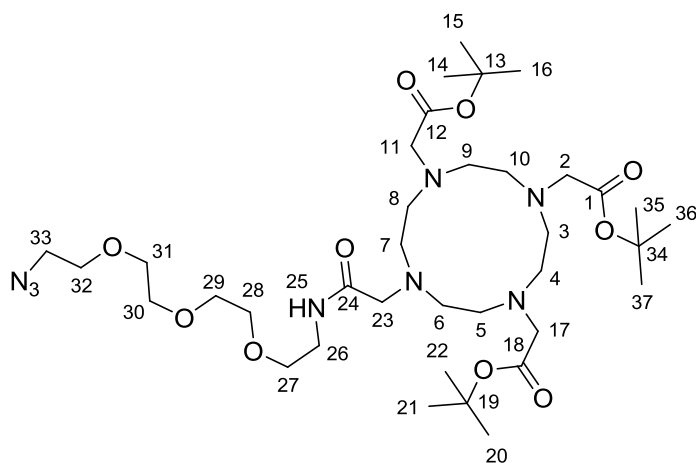
3.2.50. Compound **131**¹⁹⁷

Name: *tert*-Butyl 2-(7-[[2-(2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethyl)carbamoyl]methyl]-4,10-bis[2-(*tert*-butoxy)-2-oxoethyl]-1,4,7,10-tetraazacyclododecan-1-yl)acetate

Chemical formula: C₃₆H₆₈N₈O₁₀

Exact mass: 772.5058

Molecular weight: 772.97



Following the procedure for **129**, carboxylic acid **113** (50 mg, 87 μmol, 1.0 equiv.), HOBt (14 mg, 105 μmol, 1.2 equiv.), HBTU (36 mg, 96 μmol, 1.1 equiv.), DIPEA (25 μL), CH₂Cl₂ (1.0 mL) and commercially available amine **77** (26 μL, 131 μmol, 1.5 equiv.) afforded 35.5 mg (46 μmol, 53%) of the title compound as a colourless oil after purification by flash column chromatography; *R_f* 0.08 (3% methanol in methylene chloride); *v*_{max}/cm⁻¹ 3418 (w, N–H), 2976 (w), 2826 (w), 2103 (w, N₃), 1726 (s, C=O ester), 1676 (m, C=O amide), 1227 (s), 1159 (s), 1106 (s), 835 (vs); *δ*_H (400 MHz, CDCl₃) 6.28 (1H, t, *J* = 5.5 Hz, H-25), 3.73 – 1.72 (40H, m, H-2, H-3, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-17, H-23, H-26, H-27, H-28, H-29, H-30, H-31, H-32 and H-33), 1.39 (9H, s, H-35, H-36 and H-37), 1.39 – 1.37 (18H, s, H-14, H-15, H-16, H-20, H-21 and H-22); *δ*_C (101 MHz, CDCl₃) 172.6 (s, C-12 and C-18), 172.4 (s, C-1), 171.7 (s, C-24), [81.81, 81.75] (s, C-13, C-19 and C-34), [70.6, 70.50, 70.47, 70.3, 69.9, 69.4] (t, C-27, C-28, C-29, C-30, C-31 and C-32), 56.0 (t, C-3, C-4, C-5, C-6, C-7, C-8, C-9 and C-10), 55.7 (t, C-2), 55.6 (t, C-11 and C-17), 53.4 (t, C-23), 50.7 (t, C-

33), 39.2 (t, C-26), 28.0 (q, C-14, C-15, C-16, C-20, C-21 and C-22), 27.92 (q, C-35, C-36 and C-37).; *m/z* (ESI) (rel. int.) 795 (MNa⁺, 55), 776 (10), 775 (40), 774 (MH⁺, 100) [Found MH⁺, 773.5139. C₃₆H₆₈N₈O₁₀ requires MH⁺, 773.5137; Δ = 0.3 ppm].

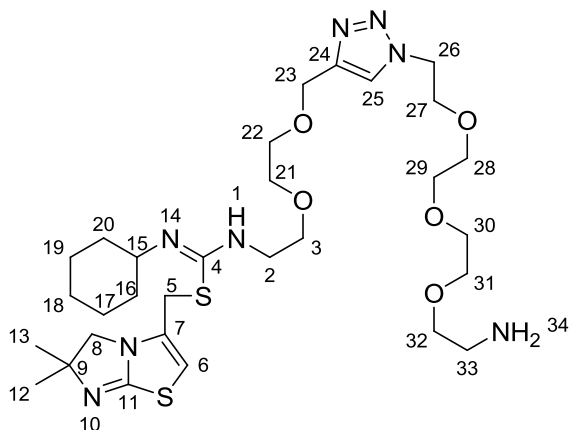
3.2.51. Compound **132**

Name: 2-(2-{[1-(2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethyl)-1*H*-1,2,3-triazol-4-yl]methoxy}ethoxy)-*N*-[(cyclohexylimino)[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl]sulfanyl]methyl]ethan-1-amine .xTFA

Chemical formula: C₃₀H₅₂N₈O₅S₂

Exact mass: 668.3502

Molecular weight of M.4TFA: 1125.01



Following the procedure for **69**, alkyne **68** (10.0 mg, 12.6 μ mol, 1.0 equiv.), commercially available azide **77** (3.75 μ L, 18.9 μ mol, 1.5 equiv.) in MeOH (250 μ L), sodium acetate buffer (250 μ L, 250 mM, pH 5), CuSO₄.5H₂O (0.63 mg, 2.5 μ mol, 20 mol%) and sodium ascorbate (11.1 mg, 63 μ mol, 5.0 equiv.) afforded 5.8 mg (5.16 μ mol, 41%) of the title compound as a colourless glass after purification by preparative HPLC, gradient 5–50% B over 20 min; $\nu_{\text{max}}/\text{cm}^{-1}$ 2873 (w, C–H), 1675 (vs, C=N⁺), 1201 (vs), 1127 (vs, CH₂–O–CH₂); δ_{H} (400 MHz, MeOH-*d*₄) 8.06 (1H, s, H-25), 4.66 – 4.55 (4H, m, H-8 and H-23), 4.09 (2H, s, H-5), 4.05 – 3.93 (2H, m, H-2), 3.93 – 3.89 (2H, m, H-26), 3.83 – 3.53 (18H, m, H-3, H-21, H-22, H-27, H-28, H-29, H-30, H-31 and H-32), 3.46 – 3.35 (1H, m, H-15), 3.13 (2H, t, *J* = 4.9 Hz, H-33), 2.07 – 1.63 (5H, m, H-16, H-17, H-18, H-19 and H-20), 1.63 – 1.51 (6H, m, H-12 and H-13), 1.51 – 1.12 (5H, m, H-16, H-17, H-18, H-19 and H-20); δ_{C} (101 MHz, MeOH-*d*₄) 125.6 (d, C-25), 89.2 (s, C-7), [70.2, 70.1, 70.0, 69.93, 69.87, 69.85] (t, C-3, C-21, C-22, C-27, C-28, C-29, C-30 and C-31), [69.0, 68.9] (t, C-26), 67.6 (s, C-9), 66.5 (t, C-32), 63.3 (t, C-23), 60.4 (d, C-15), 50.0 (t, C-8), 45.3 (t, C-2), 39.2 (t, C-33), 35.7 (t, C-5), 31.7 (t, C-16 and C-20), [26.6, 26.4] (q, C-12 and C-13), 24.5 (t, C-17, C-18 and C-19); δ_{F} (376 MHz, MeOH-*d*₄) –

Experimental procedures

76.5 (s, TFA); *m/z* (ESI) (rel. int.) 709 (60), 669 (MH⁺, 30), 241 (100) [Found MH⁺, 669.3569. C₅₈H₁₀₂N₁₂O₁₂S₂ requires MH⁺, 669.3580; Δ = −1.6 ppm].

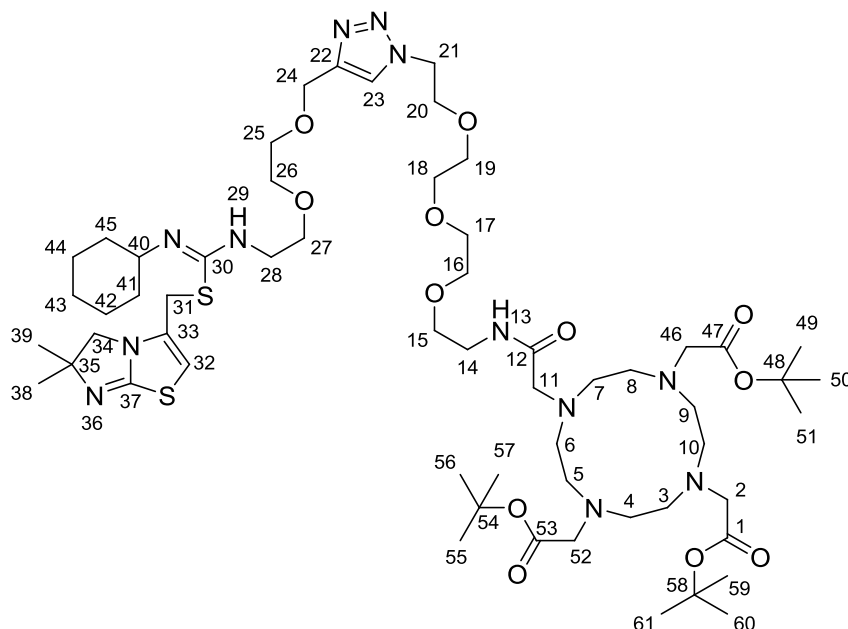
3.2.52. Compound **133**

Name: *tert*-Butyl 2-{4,10-bis[2-(*tert*-butoxy)-2-oxoethyl]-7-[(2-[2-(2-{4-(*N*-cyclohexyl-1-{2-[2-({[(6,6-dimethyl-5*H*,6*H*-imidazo[2,1-*b*][1,3]thiazol-3-yl)methyl)sulfanyl]methanimidoyl}amino)ethoxy]ethoxy)methyl)-1*H*-1,2,3-triazol-1-yl]ethoxy]ethoxy)ethoxy]ethyl} carbamoyl)methyl]-1,4,7,10-tetraazacyclododecan-1-yl}acetate .xTFA

Chemical formula: C₅₈H₁₀₂N₁₂O₁₂S₂

Exact mass: 1222.7182

Molecular weight of M.7TFA: 2021.80



To a solution of carboxylic acid **113** (26.5 mg, 46.2 μmol, 3.1 equiv.), HOBt (7.3 mg, 54.1 μmol, 3.7 equiv.), HBTU (19.2 mg, 50.7 μmol, 3.4 equiv.) and DIPEA (25.6 μL, 147 μmol, 10 equiv.) in CH₂Cl₂ (500 μL) was added a solution of amine **132** (16.5 mg, 14.7 μmol, 1.0 equiv.) in CH₂Cl₂ (500 μL) and the reaction mixture was allowed to stir at r.t. overnight before concentrating *in vacuo*. The crude residue was dissolved in H₂O + 0.1% TFA (15 mL) and purified by preparative HPLC, gradient 5–50% B over 20 min to afford 18.0 mg (8.90 μmol, 61%) of the title compound as a colourless glass; *v*_{max}/cm^{−1} 3248 (w, ar C–H), 2931 (m, C–H), 2857 (w, Cy C–H), 1703 (w, C=O); *δ*_H (400 MHz, MeOH-*d*₄) 8.11 – 8.01 (1H, s, H-23), 4.82 – 2.60 (55H, m, H-2, H-3, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-14, H-15, H-16, H-17, H-18, H-19, H-20, H-21, H-24, H-25, H-26, H-27, H-28, H-31, H-

34, H-40, H-46 and H-52), 2.15 – 1.10 (43H, m, H-38, H-39, H-41, H-42, H-43, H-44, H-45, H-49, H-50, H-51, H-55, H-56, H-57, H-59, H-60 and H-61); δ_C (101 MHz, MeOH- d_4) 171.1 (s, C-1), 170.9 (s, C-47 and C-53), 169.7 (s, C-30), 161.1 [s (q, $J = 35.5$ Hz), CF₃CO₂H], 143.9 (s, C-22), 124.5 (d, C-23), 116.6 [s (q, $J = 292.3$ Hz), CF₃CO₂H], 103.0 (d, C-32), 89.2 (s, C-33), [70.9, 70.2, 70.09, 70.05, 69.92, 69.88] (t, C-15, C-16, C-17, C-18, C-19, C-20, C-25, C-26 and C-27), 69.0 (t, C-21), 67.5 (s, C-35), 63.2 (t, C-24), 60.4 (d, C-40), 55.4 (t, C-2, C-46 and C-52), 55.0 – 50.8 (broad signal) (t, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10 and C-11), 49.9 (t, C-34), 45.3 (t, C-28), 44.0 (t, C-14), 35.7 (t, C-31), 31.6 (t, C-41 and C-45), 27.12 (q, C-49, C-50, C-51, C-55, C-56 and C-57), 27.08 (q, C-59, C-60 and C-61), [26.5, 26.4] (q, C-38 and C-39), 24.5 (t, C-43), 24.4 (t, C-42 and C-44); δ_F (376 MHz, MeOH- d_4) –76.9 (TFA); m/z (ESI) (rel. int.) 1246 (MNa⁺, 5), 1226 (1), 1225 (3), 1224 (MH⁺, 5), 595 (30), 573 (100), 517 (40) [Found MH⁺, 1223.7240. C₅₈H₁₀₂N₁₂O₁₂S₂ requires MH⁺, 1223.7260; $\Delta = -1.6$ ppm].

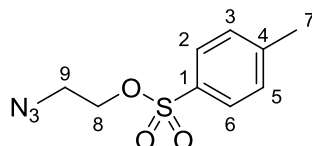
3.2.53. Compound **134**¹⁹⁸

Name: 1-[(2-Azidoethoxy)sulfonyl]4-methylbenzene

Chemical formula: C₉H₁₁N₃O₃S

Exact mass: 241.0521

Molecular weight: 241.27



Following a procedure reported in the literature,¹⁹⁹ ethylene glycol ditosylate (3.7 g, 10 mmol) was dissolved in 20 mL of anhydrous DMF, to which NaN₃ (0.65 g, 10 mmol) was added in small portions. After stirring for 10 h at r.t. under an atmosphere of N₂, the reaction mixture was diluted with water and extracted with EtOAc. The organic layer was dried over MgSO₄ and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography to afford 392 mg (1.62 mmol, 16%) of the title compound as a light yellow oil; R_f 0.22 (20% ethyl acetate in petroleum ether); δ_H (400 MHz, CDCl₃) 7.83 (2H, d, $J = 8.3$ Hz, H-2 and H-6), 7.38 (2H, d, $J = 8.6$ Hz, H-3 and H-5), 4.17 (2H, t, $J = 5.1$ Hz, H-8), 3.50 (2H, t, $J = 5.1$ Hz, H-9), 2.47 (3H, s, H-7); δ_C (101 MHz, CDCl₃) 145.3 (s, C-4), 132.6 (s, C-1), 130.0 (d, C-3 and C-5), 128.0 (d, C-2 and C-6), 68.1 (t, C-8), 49.6 (t, C-9), 21.7 (q, C-7); m/z (CI) (rel. int.) 276 (MNH₃NH₄⁺, 60), 261 (5), 260 (10), 259 (MNH₄⁺, 100) [Found MNH₄⁺, 259.0867. C₉H₁₁N₃O₃S requires MNH₄⁺, 259.0865; $\Delta = 0.8$ ppm].

4. Radiochemistry

4.1. Radiolabelling

4.1.1. Preparation of [^{68}Ga]**22**

Into a 5 mL Wheaton vial sealed with a PTFE/Silicon septum equipped with a vent needle and containing a solution of **21** (50 μg , 14.9 nmol) in sodium acetate buffer (0.5 M, pH 5.8, 2 mL) was eluted [^{68}Ga] GaCl_3 from the generator (typically 60–70 MBq in ~ 2 mL of 0.1 M aqueous HCl). The reaction mixture was allowed to react at 90 $^\circ\text{C}$ for 5 min. Analytical HPLC of the reaction mixture showed a $90\pm 6\%$ conversion ($n = 19$). The reaction mixture was then diluted with 10 mL PBS and loaded through a Waters Light C18 cartridge pre-conditioned with 5 mL of EtOH, 20 mL of air and 10 mL of PBS sequentially. The cartridge was washed with 5 mL of PBS and dried with 20 mL of air and the tracer was eluted in 4×150 μL fractions with EtOH-PBS (7:3). The second fraction contained the highest amount of activity. The non-decay corrected yield was $52\pm 8\%$ ($n = 19$) in a total of 11 ± 1 min ($n = 19$). Analytical HPLC showed a radiochemical purity of $98\pm 2\%$ ($n = 19$) using a Hewlett Packard Series 1100 G1312A pump and Lablogic Laura 3 software equipped with a linear Hewlett Packard Series 1100 G1314A UV detector ($\lambda = 254$ nm), a Lablogic Bioscan Flowcount FC-3400 PIN diode detector and a Phenomenex Gemini C18 110 $\text{\AA}$ 5 μ 150 $\times$ 4.60 mm HPLC column; method: gradient 5–68% B over 7 min; flow rate 1.00 mL.min $^{-1}$. The labelled peptide was stable in 10% EtOH–PBS for at least 4 h at room temperature.

4.1.2. Preparation of [^{18}F]**27**⁷⁶

Method A. Aqueous [^{18}F]fluoride was added to a Wheaton vial sealed with a PTFE/Silicon septum, equipped with a magnetic stirbar and a vent needle and containing 5.46 mg of $\text{K}_{2.2.2}$, 1.46 mg of KHCO_3 , 423 μL of MeCN and 77 μL of H_2O . The septum was removed and the reaction mixture was allowed to stir at 80 $^\circ\text{C}$ under a flow of N_2 . Water is azeotropically removed with anhydrous 500 μL of MeCN 3–4 times. The septum was put back in place without the vent, a mixture of 0.90 mg of 2-azidoethyl tosylate (**134**) in 200 μL anhydrous MeCN was added to the Wheaton vial and the reaction mixture was allowed to stir at 80 $^\circ\text{C}$ for 15 min. The product, [^{18}F]FEA (**27**), was used crude in the labelling step. Analytical HPLC showed a radiochemical purity $>97\%$.

Method B. 2- ^{18}F Fluoroethylazide¹⁴⁵ was synthesized on a modified GE Fastlab from 740 MBq (20 mCi) of ^{18}F fluoride in $47\pm 7\%$ DC RCY ($n = 6$) affording a solution of the product in $\sim 300\ \mu\text{L}$ MeCN in 40 min. Analytical HPLC showed a radiochemical purity of $>99\%$. Briefly, ^{18}F fluoride is trapped on a QMA cartridge and release with a solution of $\text{K}_{2.2.2}$ and KHCO_3 into the reaction vial. The mixture is dried under a flow of N_2 at $105\ ^\circ\text{C}$ and then a solution of 2-azidoethyl tosylate (**134**) is added to the reaction mixture, which is allowed to stand at $80\ ^\circ\text{C}$. The product, ^{18}F FEA (**27**), is then distilled off the reaction vessel into a Wheaton vial. Analytical HPLC showed a radiochemical purity $>99\%$.

Method C. 2- ^{18}F Fluoroethylazide¹⁴⁵ was synthesized semi-manually on a Advion Nanotek platform followed by an in-house developed automated system consisting of an eight two-way valve tower system, a mass flow controller, an oven and a six-port/two-way HPLC valve. Aqueous ^{18}F fluoride delivered from the cyclotron was trapped on a QMA cartridge (preconditioned with 1 mL of water) and released with a solution of $\text{K}_{2.2.2}$ (400 μL of a $12\ \text{g.L}^{-1}$ stock solution in water) and KHCO_3 (100 μL of a $18\ \text{g.L}^{-1}$ stock solution in water) into the concentrator. The mixture was dried under a flow of N_2 at $105\ ^\circ\text{C}$, MeCN (0.5 mL) was added and distillation continued. A solution of 2-azidoethyl tosylate (**134**) in 300 μL of MeCN was added and the reaction mixture delivered to an external heating block and heated at $80\ ^\circ\text{C}$ for 10 min. The product, ^{18}F FEA (^{18}F **27**), was then distilled off the reaction vessel into a Wheaton vial at $120\ ^\circ\text{C}$ under a stream of He ($20\ \text{mL.min}^{-1}$). Analytical HPLC showed a radiochemical purity $>99\%$.

4.1.3. Preparation of ^{18}F **28**

To a 1-mL Wheaton vial sealed with a PTFE/Silicon septum under N_2 were successively added CuSO_4 (12.5 μL , 238 mM in water), sodium ascorbate [12.5 μL , 262 mM in sodium acetate buffer (pH 5, 250 mM)], bathophenanthrolinedisulfonic acid disodium salt (12.5 μL , 298 mM in water), ^{18}F FEA (50 μL , in MeCN, 178 MBq) and **26** (50 μL , 15.0 mM in DMF) and the mixture was allowed to react at r.t. for 10 min. A solution of BnN_3 in MeOH (10 μL , 1.50 M) was added to the Wheaton vial and the reaction carried out 10 min under the same conditions. The reaction mixture was then diluted to 1.9 mL with water and purified by reverse phase semi-preparative HPLC using a Beckman Pump 127 and Lablogic Laura 3 software equipped with a linear Hewlett Packard Series 1100 G1314A UV detector ($\lambda = 254\ \text{nm}$) and a Lablogic Bioscan Flowcount FC-3400 PIN diode detector; injection loop 2000 μL ; method: isocratic 10% B for 1 min, gradient 10–40% over 9 min, 40–80% over

Experimental procedures

5 min, isocratic 80% for 1 min. The decay-corrected yield was $63 \pm 16\%$ ($n = 5$) in approximately 30 min, starting from 178 MBq [^{18}F]FEA. The non-decay-corrected EOS-RCYs was of $18 \pm 6\%$ ($n = 5$) in approximately 1.5 h from aqueous [^{18}F]fluoride. The purified fraction ($t_{\text{R}} = 11:00\text{--}12:20$) was diluted with water (3 mL) and $>99\%$ of the radioactivity was immobilised on Waters SepPak tC18 Light cartridge (preconditioned with 5 mL EtOH and 10 mL water). The cartridge was washed with water (5 mL), dried with air (10 mL) and the title compound was eluted with 25 mM HCl in EtOH in $8 \times 100\ \mu\text{L}$ fractions. Over 90% of the radioactivity was collected in 2 fractions. Analytical HPLC showed a radiochemical purity $>98\%$ using a Hewlett Packard Series 1100 G1312A pump and Lablogic Laura 3 software equipped with a linear Hewlett Packard Series 1100 G1314A UV detector ($\lambda = 254\ \text{nm}$), a Lablogic Bioscan Flowcount FC-3400 PIN diode detector and a Phenomenex Luna C18(2) $5\ \mu\text{m}$ $150 \times 4.6\ \text{mm}$ HPLC column.; method: isocratic 10% B for 1 min, gradient 10–40% over 9 min, 40–80% over 5 min, isocratic 80% for 1 min. The labelled peptide was stable in 10% EtOH–PBS for at least 4 h at room temperature.

4.1.4. Preparation of [^{18}F]35

Into a 3-mL Wheaton vial sealed with a PTFE/Silicon septum were successively added $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (50 μL , $35\ \text{g} \cdot \text{L}^{-1}$ in water), sodium ascorbate (50 μL , $174\ \text{g} \cdot \text{L}^{-1}$ in PBS), bathophenanthrolinedisulfonic acid disodium salt (20 μL , $25\ \text{g} \cdot \text{L}^{-1}$ in PBS), **26** (1.0 mg in 50 μL of MeCN–water (1:1)) and [^{18}F]FEA (100 μL in MeCN). The mixture was allowed to react at r.t. for 10 min. A solution of BnN_3 in MeOH (20 μL , $45\ \text{g} \cdot \text{L}^{-1}$ in MeOH) was added and the mixture left to react at $100\ ^\circ\text{C}$ for 10 min. The mixture was then quenched with $\sim 3\ \text{mL}$ of water and purified by reverse phase semi-preparative HPLC using a Gilson 121; injection loop 5000 μL ; method: isocratic 10% B for 1 min, gradient 10–40% over 18 min, 40–80% over 5 min, isocratic 80% for 1 min. The decay-corrected yield was $55 \pm 11\%$ ($n = 3$) in approximately 50 min, starting from 178 MBq [^{18}F]FEA. The non-decay-corrected EOS-RCYs was of $19 \pm 6\%$ ($n = 3$) in approximately 1.5 h from aqueous [^{18}F]fluoride. The purified fraction was diluted with water (5 mL) and $>99\%$ of the radioactivity was immobilised on Waters SepPak C18 Light cartridge (preconditioned with 5 mL EtOH and 10 mL PBS). The cartridge was washed with water (5 mL), dried with air (10 mL) and the title compound was eluted with PBS–EtOH (1:1) in $3 \times 300\ \mu\text{L}$ fractions. Analytical HPLC showed a radiochemical purity $>98\%$ using a QC Agilent 1100 series (quaternary pump with diode array UV detector); method: isocratic 10% B for 1 min, gradient 10–40% over 9 min, 40–80% over 5 min,

isocratic 80% for 1 min. The labelled peptide was stable in 10% EtOH–PBS for at least 4 h at room temperature.

4.1.5. Preparation of [^{18}F]69

To the crude solution of [^{18}F]27 obtained with method A was added a solution of 68 (5.6 mg), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.50 mg), bathophenanthrolinedisulfonic acid disodium salt (3.54 mg) in MeCN–PBS (3:4) (350 μL) followed by a solution of sodium ascorbate (5.85 mg) in water (100 μL). The reaction mixture was allowed to stir at 80 °C for 1 min and was quenched with H_2O + 0.1% TFA (9 mL). The tracer was immobilised on a set of two Light C18 cartridges in series, preconditioned with MeCN + 0.1% TFA (5 mL) and H_2O + 0.1% TFA (10 mL), sequentially. The set of two cartridges was washed with H_2O + 0.1% TFA (2 mL) and the radioactivity was eluted off the cartridges with MeCN– H_2O (1:1) + 0.1% TFA (2 mL). The eluate was diluted with H_2O + 0.1% TFA (3 mL) and purified by semi-preparative HPLC using a Beckman Pump 127 and Lablogic Laura 3 software equipped with a linear Hewlett Packard Series 1100 G1314A UV detector ($\lambda = 254 \text{ nm}$) and a Lablogic Bioscan Flowcount FC-3400 PIN diode detector; injection loop 5000 μL ; method: isocratic 20% B over 20 min. The decay-corrected yield was $33 \pm 13\%$ ($n = 3$) in approximately 40 min. The non-decay-corrected EOS-RCY was of $18 \pm 5\%$ ($n = 3$) in approximately 85 min from aqueous [^{18}F]fluoride. The purified fraction ($t_{\text{R}} = 10:00\text{--}11:00$) was diluted with PBS (4 mL) and 95% of the radioactivity was immobilised on Waters SepPak C18 Light cartridge (preconditioned with 5 mL EtOH, 20 mL air and 10 mL PBS). The cartridge was washed with PBS (2 mL), dried with air (20 mL) and the title compound was eluted with EtOH in $10 \times 100 \mu\text{L}$ fractions. Over 85% of the radioactivity was collected in 3 fractions. Analytical HPLC showed a radiochemical purity $>99\%$ using an Agilent 1200 Series system equipped with a diode array UV detector ($\lambda = 210 \text{ nm}$), a Raytest GABI star NaI detector and a Phenomenex Luna C18(2) 5 μm 150 $\times$ 4.6 mm HPLC column.; method: isocratic 20% B over 10 min and then gradient. 20–95% over 5 min The labelled tracer was stable in 10% EtOH–PBS for at least 4 h at room temperature.

4.1.6. Preparation of [^{68}Ga]Ga-110

Into a 5 mL Wheaton vial sealed with a PTFE/Silicon septum equipped with a vent needle and containing a solution of 110 (100 μg in 50 μL MeOH) in sodium acetate buffer (0.5 M, pH 5.8, 1.950 mL) was eluted [^{68}Ga]GaCl₃ from the generator (typically 45–55 MBq in $\sim 2 \text{ mL}$ of 0.1 M aqueous HCl). The reaction mixture was allowed to react at 90 °C for 15 min.

Experimental procedures

Analytical HPLC of the reaction mixture showed a $91\pm4\%$ conversion ($n = 5$). The reaction mixture was then diluted with 10 mL PBS and loaded through a Waters Light C18 cartridge pre-conditioned with 5 mL of EtOH, 20 mL of air and 10 mL of PBS sequentially. The cartridge was washed with 5 mL of PBS and dried with 20 mL of air and the tracer was eluted in a first fraction of 150 μL followed by $3\times 100\text{ }\mu\text{L}$ fractions with EtOH. The second fraction contained the highest amount of activity. The non-decay corrected yield was $27\pm5\%$ ($n = 7$) in a total of $23\pm2\text{ min}$ ($n = 7$). Analytical HPLC showed a radiochemical purity of $99\pm1\%$ purity ($n = 7$), using the same system as for [^{68}Ga]**22** with a gradient 5–95% B over 10 min. The labelled tracer was stable in 10% EtOH–PBS for at least 4 h at room temperature.

4.2. Specific activity

The non-radioactive isotopologues of the tracers were used to build UV-HPLC calibration curves, the characteristics of which are presented in the table below (Table 23).

Table 23. Calibration curve characteristics.

Tracer	[^{68}Ga] 22	[^{18}F] 28	[^{18}F] 35	[^{68}Ga] Ga-110
λ /nm	254	254	254	210
injection volume / μL	20	20	20	20
number of points	4	8	5	6
concentration range / μM	0.025–250	1.00–408	$1.80\text{--}1.12\times 10^3$	0.270–540
R^2	>0.9999	0.999	0.999	0.999
Apparent specific activity (GBq. μmol^{-1})	10.5 starting from 230 MBq of [^{68}Ga] GaCl_3	19.4 starting from 178 MBq of [^{18}F] FEA	6.7 starting from 178 MBq of [^{18}F] FEA	1.35 starting from 45–55 MBq of [^{68}Ga] GaCl_3

UV-HPLC chromatograms of [^{18}F]**27** did not show any absorbance at the compound's retention time, and therefore we were not able to measure an apparent specific activity for it.

4.3. Distribution coefficient

Distribution coefficients were determined by the “shake-flask” method as described in the literature.¹⁴⁹

Typically, to 100 μL of a solution of the purified tracer formulated in 10% EtOH in PBS was added 600 μL of pre-equilibrated PBS and 700 μL of pre-equilibrated octanol. The mixture was vortexed for 3 min before centrifugation at 15 krpm for 5 min. To each of 5 Eppendorf tubes were added 75 μL from the PBS layer, 700 μL of pre-equilibrated octanol and 625 μL of pre-equilibrated PBS. The Eppendorf tubes were vortexed and centrifuged as earlier. From the upper octanol layer, 450 μL was transferred to a gamma-counting tube with a pipette. The lower PBS layer was first transferred with a syringe fitted on a thin needle to a second Eppendorf tube from which 450 μL was transferred to a gamma-counting tube with a pipette. The gamma-counting tubes were placed in a gamma counter and analysed for 1 min each.

The logarithms of the distribution ratio at physiological pH between *n*-octanol and phosphate buffered saline (PBS) for our tracers was calculated and are reported in the table below.

Table 24. Logarithm of the distribution ration at physiological pH between *n*-octanol and PBS ($n = 5$).

Tracer	[⁶⁸ Ga]22	[¹⁸ F]28	[¹⁸ F]35
$\log D_{\text{pH } 7.4}$	-3.58 ± 0.15	-0.11 ± 0.02	-2.30 ± 0.30

5. Biology

The following work (except metabolite study) was performed by Liz Stevens (Imperial College London). A selection of the procedures she performed and that we believe relevant for the understanding of the results of this thesis is reported below.

5.1. In vitro work

5.1.1. Binding assays

Method A. Used for tetradecapeptide analogues.

Human leukemic T-cells Jurkat were suspended in binding buffer [PBS containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM CaCl_2 , 5 mM MgCl_2 , 0.3 mM NaN_3 , 0.5% bovine serum albumin (BSA); pH 7.4] at a concentration of 2×10^6 cells/mL. To each tube, 50 μL of antagonist solution prepared to the final concentration indicated were added, along with 50 μL [^{125}I]SDF-1 α radioligand (PerkinElmer, USA) to give a final concentration of 0.1 nM. Finally, 100 μL of the cell mixture [1.5×10^5 cells] was added to give a total assay volume of 200 μL . The tubes were incubated at room temperature for 90 min. The bound and unbound radioactivity was separated by filtration using a vacuum manifold, followed by three washes in binding buffer. The filters were punched out and counted using an NE1600 gamma counter (Thorn-EMI/Nuclear Enterprises, UK) for 240 s. The bound radioactivity was represented as a percentage of the IC_{max} using Prism 5 (GraphPad, USA). The IC_{50} curves shown are representative of three independent experiments.

Method B. Used for cyclopentapeptide and small molecule analogues.

U87.CD4.CXCR4 cells were aspirated with EDTA and incubated at 37 °C in complete media for 2 h to recover, then resuspended in binding buffer [PBS containing 2 mg/mL BSA] at a concentration of 1.5×10^6 cells/mL. To each tube, 100 μL of this cell mixture was added [1.5×10^5 cells], along with 10 μL of antagonist prepared in Me_2SO to the final concentration indicated, and [^{125}I]SDF-1 α radioligand is added to give a final concentration of 0.1 nM in a 200 μL total assay volume. The tubes were then incubated on ice for 60 min, with frequent agitation. Bound and unbound radioactivities were separated by filtration using a vacuum manifold, followed by three washes in assay buffer. The filters were then punched out and counted in a gamma counter for 240 s. The bound radioactivity was expressed as a percentage of the maximum bound radioactivity using GraphPad Prism 5.

5.1.2. Migration assays

Human leukemic T-cells Jurkat cells were serum-starved in 1% FBS – Roswell Park Memorial Institute (RPMI) medium for 24 h prior to the commencement of the experiment.

The lower well of the 24-well transwell plate with a polycarbonate membrane of 8 μm pore diameter (BD Biosciences, USA) was filled with medium (1% FBS–RPMI) containing either no additives or 100 ng/mL SDF-1 α (R&D Systems, USA). The upper wells were filled with 1×10^5 Jurkat cells, which had been either untreated or pre-treated with the indicated concentration of antagonist for 30 min (1% FBS–RPMI medium). The plates were incubated for 3 h in a humidified atmosphere at 37 °C and 5% CO₂. Following incubation, the medium from the lower wells was collected and the numbers of cells in each well counted and expressed as a percentage of the maximum. All conditions were repeated in triplicate, data shown are representative of three independent experiments.

5.1.3. Cellular uptake assay

Method A. Used for tetradecapeptide radiotracer, [⁶⁸Ga]**22**.

U87.CD4 and U87.CD4.CXCR4 cells were seeded 24 h prior to the experiment and then the appropriate wells treated with the indicated concentration of competitive inhibitor or plain medium, followed by a 30 min incubation at 37 °C 5% CO₂. The cells were then incubated with 0.37–0.74 MBq of [⁶⁸Ga]**22** for 60 min. Following incubation, the medium was removed and the cells washed twice with ice-cold PBS. After washing, the cells were scraped in PBS, collected in Eppendorf tubes and centrifuged at 5000 rpm for 5 min. The supernatant was removed and replaced with radioimmunoprecipitation assay (RIPA) buffer and transferred to counting tubes. Bound radioactivity was immediately counted in an LKB Wallac 1282 Compugamma laboratory gamma counter (PerkinElmer, USA) and the counts per minute normalised to the protein concentration of the sample (counts/ μg). Data shown are representative of three independent experiments.

Method B. Used for cyclopentapeptide radiotracers [¹⁸F]**28** and [¹⁸F]**35** and small molecule analogue [⁶⁸Ga]**143**.

U87.CD4 and U87.CD4.CXCR4 cells were seeded 48 h prior to the experiment in complete DMEM in triplicate wells of 6-well plates at a density of 3×10^5 cells/well. At commencement of the experiment, the wells were incubated with 0.74 MBq (20 μCi) of radiotracer for 60 min at 37 °C. After incubation the unbound radioactivity was washed from the cells twice with ice-cold PBS, scraped in PBS and collected by centrifugation at 5000 rpm for 3 min at 4 °C and then counted by gamma counter. The decay-corrected counts per minute was normalised to the protein content of the wells using GraphPad Prism 5.

5.2. In vivo work

5.2.1. Animal experiments

All animal work was performed by licensed investigators in accordance with the United Kingdom's *Guidance on the Operation of Animals (Scientific Procedures) Act 1986* and within guidelines set out by the United Kingdom National Cancer Research Institute Committee on Welfare of Animals in Cancer Research.²⁰⁰

5.2.2. Xenotransplantation

Female BALB/c nu/nu athymic nude mice aged between 6 and 8 weeks were obtained from Charles River (USA). Xenotransplants were performed under 2.5% isoflurane anaesthesia, by injection of the specified cell line (4.5×10^6 cells in 100 μ l PBS) subcutaneously to the nape of the neck. Tumour growth was monitored by callipers and experiments begun once the tumours reached at least 80 mm³.

5.2.3. Imaging experiments and biodistribution at 60 min post-injection

The mice were anaesthetised with 2.5% isoflurane and placed in a thermostatically controlled rig in a dedicated small animal CT/PET scanner (Siemens Multimodality Inveon, Siemens Molecular Imaging Inc., USA). A CT orientation scan was performed prior to a dynamic PET scan acquired over 60 min in a list-mode format, following a 3.7 MBq (100 μ Ci) injection of tracer *via* a lateral tail vein cannulae. Cumulative images of the dynamic data at 60 min were reconstructed using an iterative 3D ordered subset expectation maximisation (OSEM3D) reconstruction algorithm, also comprising filtered back-projection, signal attenuation and 3D reconstruction algorithms. Siemens Inveon Research Workplace software was used to identify 3D regions of interest (ROIs) using a combination of the reconstructed iterative image, and the CT orientation scan. The count densities (counts/mL) of these ROIs were averaged for each of the 19 time points to obtain time *versus* activity curves (TACs) for each ROI. Tumour TACs were normalised to the injected dose to obtain the normalised uptake value (NUV) expressed as %ID/mL. Biodistribution experiments were conducted immediately after imaging experiments had finished; Whilst under anaesthesia (2.5% isoflurane) the animals were sacrificed by cardiac puncture and the specified tissues harvested and counted for radioactivity in the LKB Wallac 1282 Compugamma laboratory gamma counter (PerkinElmer, USA). The decay-corrected counts per minute was normalised to the calculated

injected dose and to the weight of the counted tissue, to express the biodistribution of radiotracer in the specified tissue as %ID/g.

5.2.4. *Dynamic biodistribution*

Female BALB/c mice aged between 6 and 8 weeks were obtained from Charles River (USA), anaesthetised with 2.5% isoflurane and injected with 8.14–10.4 MBq (220–280 μ Ci) of tracer *via* a lateral tail vein cannulae. Whilst under anaesthesia the animals were sacrificed by cardiac puncture at the indicated time post-injection and the specified tissues harvested and counted for radioactivity in the LKB Wallac 1282 Compugamma laboratory gamma counter (PerkinElmer, USA). The decay-corrected counts per minute was normalised to the calculated injected dose and to the weight of the counted tissue, to express the biodistribution of radiotracer in the specified tissue as %ID/g. Additional samples of plasma, liver and urine were collected and stored on ice for the metabolite study.

5.2.5. *Metabolite study*

To the urine and plasma samples were added 100 μ L of an aqueous solution saturated with urea at 37 °C and this mixture was added dropwise to an equivalent volume of ice-cold MeOH. The mixture was vortexed for 30 s and centrifuged (15 krpm, 5 min, 4 °C). The supernatant was added to an equivalent volume of ice-cold MeOH, and the mixture was vortexed and centrifuged as earlier. To each liver sample were added 100 μ L of an aqueous solution saturated with urea at 37 °C and 1 mL of ice-cold MeOH and the sample was homogenised with an IKA Ultra-Turrax® T25 basic homogeniser at 25000 min⁻¹. The resulting suspension was centrifuged (15 krpm, 5 min, 4 °C). The supernatant of each sample preparation was added to 3 mL of ice-cold H₂O + 0.1% TFA. This mixture was filtered through a 0.20 μ m Millipore Millex®-LG 0.20 μ m $\times$ 13 mm PTFE syringe-driven filter unit and injected into the HPLC using an Agilent 1100 Series G1312A pump and Lablogic Laura 3 software equipped with a linear Agilent 1100 Series G1314A UV detector (λ = 254 nm), a IN/US Systems γ -RAM model 3 detector and a Waters μ Bondapak C18 125 Å 10 μ 7.8 $\times$ 300 mm HPLC column; injection loop 5000 μ L; mobile phase (A) H₂O + 0.1% TFA and (B) MeCN + 0.1% TFA; method: gradient 5–65% B over 15 min. Flow rate 5.00 mL.min⁻¹.

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