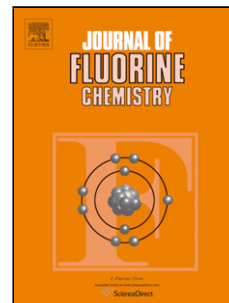


Accepted Manuscript

Title: Strategies for radiolabelling antibody, antibody fragments and affibodies with fluorine-18 as tracers for positron emission tomography (PET)

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PII: S0022-1139(17)30252-X
DOI: <http://dx.doi.org/doi:10.1016/j.jfluchem.2017.08.001>
Reference: FLUOR 9027

To appear in: *FLUOR*

Received date: 4-6-2017
Revised date: 2-8-2017
Accepted date: 3-8-2017

Please cite this article as: Joshua Clark, David O'Hagan, Strategies for radiolabelling antibody, antibody fragments and affibodies with fluorine-18 as tracers for positron emission tomography (PET), Journal of Fluorine Chemistry <http://dx.doi.org/10.1016/j.jfluchem.2017.08.001>

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Strategies for radiolabelling antibody, antibody fragments and affibodies with fluorine-18 as tracers for positron emission tomography (PET)

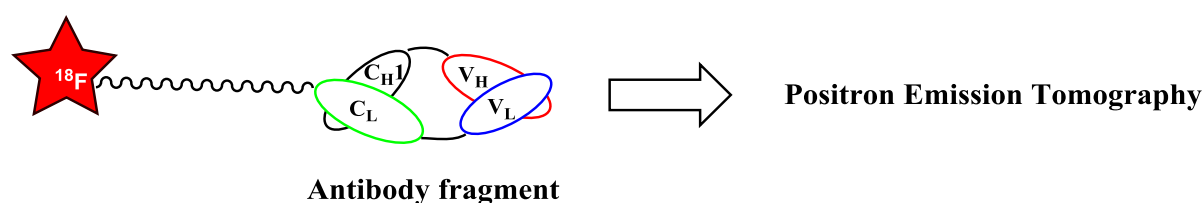
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Graphical abstract



Abstract

The use of fluorine-18 as a radionuclide for positron emission tomography (PET) has become increasingly popular over the last two decades and cancer and neurology clinical centres worldwide are increasingly establishing competence in this modality for diagnostic imaging. Progress has been particularly impressive for small molecule pharmaceutical candidates and low molecular weight affinity peptides, where clearance rates of the peptides in the body are compatible with the half-life of fluorine-18 ($t_{1/2} = 110$ min). However high molecular weight proteins present challenges as they circulate and clear much more slowly. This review focuses on the methods used to radiolabel antibodies and their derivatives with fluorine-18 as tracers for PET. The very high specificity of these biomolecules for disease indicators at the molecular level makes labelling them attractive, however antibodies can circulate for days within the blood, with slow clearance times due to their high molecular weights, and this is

inconsistent with the relatively short half-life of fluorine-18. Thus, lower molecular weight fragments of antibodies present more realistic targets for labelling. This review describes the approaches and protocols which have been successfully used to radiolabel antibodies and particularly antibody fragments with fluorine-18, and highlights this challenging aspect of fluorine-18 labelling for PET imaging.

Keywords: fluorine-18, positron emission tomography, antibody, antibody fragment, bioconjugation.

1. Introduction

Positron emission tomography (PET) is a widespread imaging modality for clinical applications such as: disease diagnosis, tumour detection and brain imaging [1]. The fluorine-18 isotope is particularly suitable for use as a positron emitter owing to its half-life ($t_{1/2} = 110$ min) which is long enough to achieve relatively elaborate synthetic sequences from cyclotron generated fluoride-18. When compared to other commonly used radionuclides; oxygen-15 ($t_{1/2} = 2$ min), nitrogen-13 ($t_{1/2} = 10$ min) and carbon-11 ($t_{1/2} = 20$ min), the longevity of fluorine-18 represents a clear advantage. Furthermore, the positron emission energy of fluorine-18 is weaker than all other first row positron emitters which optimises resolution in imaging [2,3]. On the other hand, the half-life of fluorine-18 is short enough to achieve a high signal to noise ratio on a timescale which complements the pharmacokinetic profile of pharmaceutical type molecules of interest, in contrast to more persistent heavier radionuclides [4].

Consequently, there is a mature research activity focussed on radiolabelling with the fluorine-18 isotope. To date the focus has been on low molecular weight drug like molecules and extending to bioactive peptides with molecular weights in the 1-4 kDa range. The area has been the subject of a number of recent comprehensive reviews [5–9]. With the advent of antibody engineering technologies, there has been a steady interest in exploring technology for the radiolabelling of antibodies for clinical imaging, due to their exquisite affinity to molecular markers of disease on cell surfaces. Early reports tended to focus on the labelling of whole antibodies (160 kDa) but clinical applications have been hindered by slow circulation kinetics resulting from their high molecular weights [10]. Typically, antibodies circulate in the blood for several days rendering them incompatible with fluorine-18 due to a disparate shorter half-life. As a result, efforts to radiolabel smaller antibody fragments (25-110 kDa), which circulate much more rapidly, have been pursued and with more success. These fragments are composed of various derivatives of their parent antibodies (Fig. 1.) but

universally incorporate an antigen-binding site to retain binding affinity. To reduce molecular weight further the radiolabelling of even smaller non-antibody, antigen-targeting molecules known as affibodies (6.5-14 kDa) has been explored and these possess additional advantages such as chemical stability [11].

The limited half-life of fluorine-18 demands that isotopic incorporation into the biomolecule is completed as fast as possible to prevent excess radioactive decay. The fluorine-18 isotope is generated in a cyclotron wherein the bombardment of oxygen-18 enriched water [$^{18}\text{OH}_2$] with high energy protons generates an aqueous solution of fluoride-18 in pico- to nano-molar concentrations [3]. Difficulty arises when attempting to utilise this solution for labelling because of the poor nucleophilicity of aqueous fluoride [12]. Methods generally rely on a drying step wherein water is removed and fluoride-18 is fixed as its potassium salt in an aprotic organic solvent with a phase transfer catalyst (usually kryptofix) to bind K^+ . The resulting organic solution containing soluble fluoride-18 ion is then used to radiolabel a prosthetic group which can be selectively conjugated to a desired moiety on the biomolecule [13]. Recently efforts have been made to circumvent the need for drying entirely. These strategies include; exploitation of the labile B(boron)-F bond to facilitate isotopic exchange in aqueous media [14], the use of chelating ligands to trap Al(aluminium)-fluorine-18 [15], aqueous inverse electron demand Diels-Alder (IEDDA) reactions [16] and enzymatic methods [17]. Also of note are pre-targeting strategies which typically involve the injection of an antibody-streptavidin construct several days prior to administration of a low molecular weight fluorine-18 tagged biotin [18].

Antibody and antibody fragments {Note to Editor: This section should appear as an information inset}

Antibodies are large proteins (immunoglobulins - Igs) which have different functions in the immune system. Immunoglobulin G (IgG) is the most abundant (Fig. 1). Structurally they have two heavy (H) and two Light (L) polypeptide chains, held together through disulphide bridges. The H and L chains can be divided into variable (V) and constant (C) domains. The variation in the V_H and V_L chains determines the unique antigen-recognition site. IgG has a high molecular weight of 160 kDa, resulting in its persistence in the circulatory system for several days after injection. However, molecular weight fragments of the antibody present better candidates for radiolabelling as their uptake and clearance times are more rapid. In this review radiolabelling of several categories of engineered antibody fragments are

highlighted. This inset provides an indication of the topology of the different fragment categories.

The Fc region of antibodies is not associated with antigen binding but fulfils a number of biological roles; it binds to receptors on target cells and confers effector functions which result in the destruction of the targeted cell [19,20]. The Fab region is the antigen-binding domain and is therefore integral to biotechnological applications. The region consists of two constant domains (the light domain C_L and the heavy domain C_{H1}) and two variable domains (the light domain V_L and the heavy domain V_H) the latter of which is responsible for the unique specificity of the antigen binding site. The antibody fragments discussed here will include; the $F(ab')_2$ homodimer fragment (~110 kDa), the Fab' fragment (~55 kDa), the Fab fragment (~50 kDa) and the Fv/scFv fragment (~25 kDa). Diabodies (~55 kDa) are non-covalent dimers of the Fv fragment ($(Fv)_2$) or the scFv fragment ($(scFv)_2$), in the case of cyst-diabodies a free thiol moiety is present which leads to disulphide-stabilisation. The $(Fab')_2$ homodimer consists of both Fab regions linked by disulphides and therefore retains the entire binding region of the antibody. The Fab' fragment is derived from the homodimer by enzymatic reduction and retains a free thiol group. In contrast, the Fab fragment does not retain the thiol moiety and consists only of the four domains (C_L , C_{H1} , V_L and V_H) linked by disulphide interactions. In contrast Fv and scFv consist only of the two variable domains (V_L and V_H) and in scFv fragments these variable domains are linked by a hydrophilic peptide chain consisting of about 15 amino acid residues. Similarly a dsFv fragment is based on Fv but with an interchain disulphide bond [20,21]. A promising class of engineered proteins are affibodies which combine the recognition properties of antibodies with several desirable traits including small size (6.5-14 kDa) and increased stability to heat and harsh chemical conditions. Affibodies consist of 58 amino acids arranged into three helices. Of these, 13 residues may be subject to mutagenesis to determine the specificity of binding. These residues have been varied combinatorially to provide extensive libraries of affibodies each with a unique binding site on a target antigen [22][23].

Another class of Immunoglobulin G is the camelid-derived heavy-chain variant which possesses a non-binding region as found in the Fc region of conventional IgG but a distinct binding region consisting only of a single variable domain V_{HH} . The V_{HH} fragment is also known as a nanobody, reflecting its relative low molecular weight (12-15 kDa) which aids its biotechnological application. Furthermore, nanobodies possess properties such as low immunogenicity and high stability which make them particularly suitable probes [24].

{Note to Editor: End inset here}

2. Strategies for fluorine-18 labelling of antibodies and their fragments

Early methods for the radiolabelling of antibodies and derivatives utilised radiolabelled small molecules for conjugation (Fig. 2). The first application of fluorine-18 for antibody labelling was reported in 1987 when the alkylating agents **7** and **8** were used to radiolabel the antibody IgA (162 kDa) [25]. The synthesis of **7** (20-33% yield, 45-50 min) was achieved from S_NAr of 3,5-dinitrobenzotrile no carrier added (n.c.a.) fluoride-18, followed by the addition of sodium methoxide. The synthesis of **8** (28-40% yield, 75 min) also began with an S_NAr utilising n.c.a. fluoride-18 with 4-nitrobenzotrile and was elaborated to the final product by addition of MeLi and CuBr₂. Antibody conjugation was conducted under ambient aqueous conditions (borate buffer, pH 8.0, 47 °C, 1 h). The radiochemical yields (RCY) for the labelling of IgA were 16% for **7** and 46% for **8**.

Later, **8** was utilised to label the F(ab')₂ fragment of the murine BB5-G1 antibody (specific for a human parathyroid antigen) in 10% yield (from **8**) [26]. The labelled fragment displayed specificity of 10:1 parathyroid-to-muscle after 4h in nude mice models.

Later the mouse Mel-14 antibody (Fab')₂ fragment was radiolabelled with fluorine-18 [13,27,28]. Radiolabelling was achieved using **1**, which was synthesised by S_NAr of **10** to give **6** (Scheme 1). Subsequently, oxidation and N-hydroxysuccinamide (NHS) coupling were used to obtain **1** (100 min, RCY 25%). Conjugation was achieved under ambient aqueous conditions (pH 8.5 borate buffer, 15-20 min) in a 40-60% yield which was an improvement relative to the previously described methods involving **7** and **8**. *In vitro* binding and mouse tissue distribution studies showed similar results to those observed for the antibody fragment tagged with the ¹²⁵I analogue of **1**. Later refinements to the method reduced the synthesis and purification time of **1** to about 45 mins [29]. These included an increased temperature for all steps and the use of dicyclohexyl carbodiimide (DCC) as the coupling promoter.

Additional early work utilised the prosthetic group **3** [30–34]. The synthesis of **3** usually begins with an S_NAr reaction of **11** with tetrabutylammonium [¹⁸F]fluoride to give **12**. Amide coupling with **12** gives **3** (Scheme 2). Conjugation to the Mel-14 F(ab')₂ and the TP-3 Fab fragments were achieved under analogous conditions as developed for **1**. A comparative study showed that the total synthesis time (85 min) for **3** was shorter than that (95 min) for **1**, but that the overall radiochemical yields were similar (RCY 10-12%) [30].

Attempts to improve upon **1** as a prosthetic group continued with the exploration of **9** for radiolabelling of IgG and the IL-2 α dsFv fragment [35,36]. The synthesis of **9** is achieved by an S_N2 reaction of **13** with fluoride-18 (Scheme 3). This significantly reduced the preparation time (30-35 min) relative to **1**, although RCYs (10-20%) were modest due to the formation of a side product resulting from ester cleavage. Labelling of IgG (borate buffer, pH 8.5, 10-15 min) was achieved in a 50-70% yield. The overall RCY was 10% and accomplished in 65 min including purification. The labelling of the IL-2 α Fv fragment (borate buffer, pH 8.5, 10 min) with **9** was achieved in 33-45% yield [36–38].

Wester et al., compared **1** and **5** as tags for radiolabelling antibody IgG [12]. Their improved synthesis of **1** was achieved in a 35% RCY in 55 min (Scheme 5). Subsequent labelling of IgG was achieved (borate buffer, pH 8.5, r.t., 15 min) in 48-60% RCY. Azeotropically dried [¹⁸F]fluoride was used to convert **14** to **5** in an efficient 70% RCY in 4 min (Scheme 4). However, the labelling of IgG with **5** (15W UV lamp, 365 nm, 5 min) proceeded in a low RCY of 11%. Notably **5** is conjugated to the antibody by a photochemical reaction proceeding through a nitrene intermediate. The reduced RCY in this case was attributed to the short lifetime of the nitrene and presents a limitation for using **5**, despite its high-yielding one-step preparation from [¹⁸F]fluoride.

A comparative study by Reske et al. contrasted the use of fluorine-18 and technetium-99m as radiolabels for the leukemia relevant anti-CD66 antibody [39]. The chosen prosthetic group **1** was prepared by automated synthesis (Scheme 5) giving a decay-corrected radiochemical yield (dcRCY) of 32% in 75 min. Conjugation to anti-CD66 (borate buffer, pH 8.5, r.t., 10 min) was achieved in a 9-14% RCY. Unusually for whole antibodies, anti-CD66 is notable for its fast blood clearance and its fast uptake by bone marrow tissue [40]. Consequently, anti-CD66 is suitable for use on the fluorine-18 isotope timescale and the report indicates that the labelled antibody provided excellent PET image quality and a clear selectivity for bone marrow tissue.

Cai et al. reported the use of **1** to radiolabel the anti-carcinoembryonic (CEA) T84.66 diabody ((scFv)₂) [41]. The authors note that the use of the diabody is preferred to the scFv fragment because the former is capable of bivalent binding (relative to monovalent binding for scFv) which results in increased tumour uptake and retention while retaining a low enough molecular weight to be cleared from the blood on the fluorine-18 timescale. The synthesis of **1** (27-38%, 130-146 min) was achieved in an automated module according to standard protocols (Scheme 1). However, the labelling of T84.66 (borate buffer, pH 8.5, 40 °C, 30-40 min) was inefficient due to a limited number of accessible lysine residues on the diabody surface. As a result, the overall dcRCY was just 1.2-1.6%. The labelled diabody

showed high tumour uptake for CEA positive tumours in mouse models and high-contrast PET images were viewable within 1 h of injection.

The anti-CEA T84.66 diabody has been engineered to incorporate a terminal cysteine residue which results in a disulfide bond between the subunits of the dimer giving a cys-diabody. The cys-diabody has been radiolabelled using **1** [42,43]. The synthesis of **1** (35% RCY, 40 min) involved a one-pot microwave-assisted process adapted from Scheme 1. The cys-diabody was radiolabelled (borate buffer, pH 8.5, r.t., 30-45 min) with **1** resulting in an overall decay-corrected RCY of 1.8-2.5% in 100min.

Additionally, **1** has been used to label an anti-LIBS scFv [44]. The fragment in question binds the platelet integrin receptor IIb/IIIa and is an attractive target for the detection of activated platelets in carotid artery thrombosis. The synthesis of **1** was completed according to a previously reported protocol adapted from Scheme 1 [45,46]. Crucially, the use of an automated synthetic module facilitated improvement in dcRCY of **1**, typically 80% in 58 min. Labelling of ScFv_{anti-LIBS} (phosphate buffered saline, Ca²⁺/Mg²⁺, 30 min) proceeded in an overall RCY of 1.3-2.3%. Mice PET studies demonstrated a greater uptake for the labelled fragment at injured vessels compared to intact ones, indicating the selectivity required for diagnostic imaging of thrombosis.

The use of **1** for the labelling of an anti-MMR camelid nanobody was reported in 2015 [47]. The synthesis of **1** was automated and modular, facilitating a one pot synthesis, and with a RCY of 50-60% in 50 min (Scheme 5). Conjugation to the nanobody (borate buffer, pH 7.4-9.0) gave a decay corrected RCY of 20-30%. The tracer displayed specificity for macrophage mannose receptors (MMR) and produced the desired biodistribution in mice models.

The anti-HER2 nanobody has also been radiolabelled using **1** [48]. The automated, modular synthesis of **1** was further optimised giving a dcRCY of 60-70%. The anti-HER2 nanobody was successfully labelled (borate buffer, pH 8.4-8.5, r.t., 20 min) in a 20-30% dcRCY resulting in an overall dcRCY of 5-15% in 180 min. The tracer's specificity to the HER2-antigen was successfully shown in mice, generating high contrast PET images.

Recently **1** was used to radiolabel the scFv fragment of the anti-CA125 antibody B43.13 [49]. The target antigen is associated with epithelial ovarian cancer. The automated synthesis of **1** was adapted from Scheme 5 and gave a dc-RCY of 48-86% in 54-62 min. Radiolabelling of the scFv fragment (borate buffer, pH 8.3, 45 min, 30 °C) was achieved, resulting in an overall dc-RCY of 1.9-5.6%. In mice models a modest uptake was achieved and the authors

note that while scFv fragments are attractive for fluorine-18 PET because of their rapid blood clearance, their small size results in their rapid renal clearance. For this reason and that of increased binding efficiency the authors suggest cys-diabodies as better candidates for tumour uptake in PET.

The anti-VCAM-1 nanobody, cAbVCAM1-5 has been labelled by **1** [50]. The radiosynthesis of **1** was achieved utilising a method similar to standard protocols (Scheme 1, Scheme 5) in 50-60% RCY [47]. Conjugation (borate buffer, pH 8.5, 20 min, r.t.) to the nanobody was achieved facilitating PET studies in mice which imaged atherosclerotic lesions.

Optimisation of the conjugation between **1** and biomolecules has extended to the use of a digital microfluidic droplet generation (DMDG) chip [51]. The radiosynthesis of **1** was performed in a one-pot microwave-assisted process modified from previous procedures (Scheme 1, Scheme 5) in 30-40% RCY within 60 min. The DMDG chip facilitates precise control of conjugation mixture composition (**1**, biomolecule, buffer (variable pH)) at the nanolitre scale. The chip can therefore be used to screen a variety of conditions for a given system before scaling up the optimised protocol, specifically pH and biomolecule concentration. To demonstrate this utility the DMDG chip was used to optimise labelling conditions for the anti-PSCA A2 diabody and; a pH of 8.7, a volumetric ratio of 1:2 (1:diabody) and a diabody concentration of 1 mg/mL⁻¹ were found to give optimum labelling yields. On scale-up a RCY of ~23% was achieved in 10-20 min. MicroPET imaging of tumours in xenografted murine models were viewable within 30-240 min. The DMDG chip was further utilised for the conjugation of **1** and an anti-HER2 diabody. Optimised conditions (pH 8.6, 0.33 mgmL⁻¹) were used to obtain the conjugate in 18% RCY which facilitated microPET imaging of xenografted murine models.

Two antibody fragments that target the $\alpha_v\beta_6$ integrin (a diabody and a cys-diabody) have been radiolabelled using **1** [52]. The radiosynthesis of **1** was achieved using a standard protocol (Scheme 5) and gave 34-38.% RCY in 62-64 min. Conjugation to the diabody and cys-diabody were performed according to the DMDG optimised conditions for **1** (borate buffer, pH 8.7, 37 °C, 10 min) in 7-10% and 19-26% RCY respectively. The authors note that specific DMDG optimisation for the diabodies under investigation could improve the RCY.

Recently Vaidyanathan et al. sought to implement a 'residualising' label to extend the radioactive time profile of the target [53]. Some antigens are internalising, meaning that they become incorporated within the target cell after binding. After internalisation, proteolysis of the conjugate takes place in the lysosome. Non-residualising products from the catabolism such as [¹⁸F]fluoride, are likely to be removed quickly from the cell resulting in a loss of

radioactivity. Residualising labels are retained at the target and their catabolites can be retained within the lysozyme and are therefore less likely to be removed from the cell. Nucleophilic substitution of **19** gave **20** which was immediately exposed to **18** under CuAAC conditions. After a 'click' reaction, deprotection of the guanidinium residue yielded **21** (Scheme 6, dcRCY 5.8-11.3%, 100 min). Labelling of the anti-HER2 sdAb 5F7 (borate buffer, pH 8.5, 20 °C, 20 min) proceeded in an overall dcRCY of 2-3%. For comparison, the **1**-nanobody conjugate was prepared. Radiolabelling of the nanobody with **1** was achieved in 26-49% RCY. Crucially the **21**-nanobody conjugate displayed significantly higher accumulation and retention in target cells and xenografted mice than was observed for the **1** conjugate, consistent with the residualizing nature of **21** [54]. The use of **21** was further extended to label another anti-HER2 sdAb, 2Rs15d which binds to a different site of HER2 (making it compatible with trastuzumab/pertuzumab therapy) [55]. A similar labelling protocol gave RCYs in the range 30-59%. The resulting conjugate had a lower tumour retention *in vivo* and *in vitro* than the 5F7 conjugate.

The prosthetic group **4** has been used for the radiolabelling of the affibody $Z_{\text{EGFR}:1907}$ [56]. Beginning with **1**, amide coupling with **22** was used to synthesis **4** (Scheme 7). With **4** the affibody was labelled site-specifically through the N terminus cysteine residue in buffered conditions (pH 7, 40 min). The authors note in a later paper that the total synthesis time and yield (3h, RCY 10%) for affibody preparation placed limitations on routine use of **4**- $Z_{\text{EGFR}:1907}$ as an imaging agent [57].

Similarly, two HER2-specific affibodies $Z_{\text{HER2}:342}$ and $Z_{\text{HER2}:2395}$ were radiolabelled using **4** [58,59]. Unlike before, the synthesis of **4** proceeded via the formation of **16** which underwent diethyl cyanophosphonate-mediated amide coupling (RCY 17-27%). Conjugation (PBS, pH 7.4, r.t., 30 min) was achieved in RCY's up to 60% and in overall RCYs of between 9-13% in approximately 2 hours. The radiolabelled $Z_{\text{HER2}:342}$ was used to image xenografted mice and a retention of binding specificity was observed. The labelled affibody $Z_{\text{HER2}:342}$ has more recently been applied to monitor activity of a candidate anti-cancer drug and known HER2 down regulator, 17-DMAG [60]. The ability of the conjugate to image HER2 expressing tumours quantitatively was shown to facilitate its assessment of drug candidates. Similarly, the conjugate was utilised to quantify tumour responses to chemotherapeutic treatment with trastuzumab [61]. Animal studies indicated a lower uptake of the conjugate after administration of trastuzumab consistent with a downregulation of HER2.

The prosthetic group **6** has been utilised to radiolabel two Anti-HER2 affibody molecules [62]; the monomeric $Z_{\text{HER2}:477}$ and the dimeric $(Z_{\text{HER2}:477})_2$. The synthesis of **6** was performed using the procedure in Scheme 1 and gave the product in a dcRCY of between 50-70% in 40

min. Labelling of the affibody (ammonium acetate, pH 4, 70 °C, 15 min) was achieved resulting in an overall dcRCY of 13-18% in a total of 100 min.

The use of **6** has also been extended to two synthetic variant affibodies of Z_{HER2:342} [63]. The synthesis of **6** was performed using the method in Scheme 1 and gave the product in 50-70% RCY. The affibody fragments were successfully labelled (citrate, phosphate, MeCN, pH 2.6, 70 °C, 15 min) in an overall dcRCY of 3-5%.

Similarly, **6** has been used to radiolabel Anti-HER2 dimeric affibody via oxime ligation [11]. The radiosynthesis of **6** was as before (Scheme 1) and proceeded in 50-70% dcRCY within 40 min. The affibody was pre-functionalised to provide an aminoxy handle and was conjugated to **6** (ammonium acetate, pH 4, 70 °C, 15 min) in 26-30% dcRCY.

Another anti-HER2 affibody Z_{HER2:2891} was recently labelled with **6** [64] and conjugation (aniline hydrochloride, water, 70 °C, 20 min) followed in an overall RCY of 30%. Uptake of the conjugate was greater for HER2-positive than for HER2-negative cell lines which indicates suitability in distinguishing the more aggressive positive tumours from less aggressive negative ones.

A modular approach exploiting the CuAAC click reaction has been developed for the radiolabelling of the trastuzumab Fab fragment [65]. The triazole **26** was prepared by click reaction with **24** (Scheme 8) in 50-59% yield from the unlabelled azide **23**. Notably, **26** has the benefit of improved water-solubility relative to other prosthetic groups due to the hydrophilicity of the PEG linker which can be extended if desired. The trastuzumab Fab fragment was radiolabelled using **26** (phosphate buffer, pH 8, r.t., 10 min) with a conjugation efficiency of 23-37%. Overall the dcRCY was 10-16% which was obtained between 78-86 min. The labelled fragment was shown to be an effective PET tracer for the imaging of HER2 receptors in murine models.

Rosa-Neto et al. have developed a method for fluorine-18 labelling of bovine IgG with a silicon-containing prosthetic group, **28** [66]. The pre-assembled prosthetic group is radiolabelled by a one-step isotopic exchange with **27** to give **28** (Scheme 9) in 95% RCY. Labelling occurs by the amidation of the isothiocyanate (carbonate buffer, pH 9, r.t., 10-20 min) and gave a RCY of 40%. The overall RCY was approximately 38% obtained in a total of 40-50 min.

Another Si-based prosthetic group **30**, has been utilised to label a β -cell-specific scFv fragment [67]. The pre-assembled prosthetic group is radiolabelled in a one-step isotopic exchange with **29** to obtain pure **30** (Scheme 10) in 20-25 min and a 56% RCY. The fluorine-

18 was obtained without azeotropic drying according to the rapid Munich method which utilises a strong anion exchange (SAX) cartridge to trap aqueous fluoride-18 [67,68]. A drying step is usually required due to the poor nucleophilicity of aqueous fluoride [69]. The labelling of the scFv fragment with **30** (borate buffer, pH 9, r.t., 30 min) gave a RCY of 17-19% and can be compared to that with **1** (borate buffer, pH 9, r.t., 20 min) in a RCY of 11-17%.

A recent report details the preparation and application of a new heterocyclic prosthetic group **32** based on Barbas linkers [70]. Synthesis involved readily available precursors and the radiolabel was introduced in the final step by S_N2 reaction with **31** (Scheme 11) which gave **32** in 21-33% dcRCY in 10 min. With **32** in hand conjugation (PBS, 50 °C, 15 min) to the modified affibody Z_{HER2:2395}-Cys was achieved in 40% RCY. Notably negligible conjugate was recovered at 37 °C. The total synthesis, conjugation and purification time was about 160 min and the authors noted an unusually high specific activity of the conjugate. Additional advantages of **32** were cited as a single-step synthesis with a single product and the stability of its conjugates *in vivo*.

An attractive approach to fluorine-18 radiolabelling involves the direct use of aqueous fluoride-18 from the cyclotron. However, this method requires water soluble substrates that are sufficiently fluorophilic to sequester aqueous fluoride. Smith et al. [18], used aqueous fluoride-18 to label an antibody in a pre-targeting approach, by exploiting the biotin-avidin interaction. Beginning with **33**, radiolabelling was achieved by treatment with an acidic, aqueous solution of fluoride-18 to give **34** (Scheme 12) in 2 h and 65% yield. Following the incubation of three separate HER2 expressing cell lines with Neutraavidin™ conjugated trastuzumab **34** was added and exhibited binding to those cells. Notably the cells expressing HER2 at higher levels displayed greater binding to **34** indicating the sensitivity of the approach. These *in vitro* results indicate the potential of the pre-targeting method for clinical applications.

A similar pre-targeting approach has utilised the lability of the B-F bond to radiolabel dendron-containing **35** giving **36** which bound to HER2 cells pre-targeted with avidin-trastuzumab [71]. The radiolabel was introduced in the final step (37 °C, 1 h) utilising either a trifluoroboroaryl handle or the pinacolboronyl moiety (Scheme 13). In either case labelling efficiency was about 60%. The efficacy of the pre-targeting approach was shown *in vitro* but *in vivo* results are pending further investigation.

An alternative and more widely explored method to label antibody fragments and other biomolecules with aqueous fluoride-18 was developed by Lütje, McBride et al. [15,72],

wherein the preparation of the aluminium-fluorine-18 bound ligand **38** is described. Beginning with the unbound ligand **37**, **38** is obtained within 60 mins (Scheme 14) with a labelling efficiency of 92-96% and a high specific activity following solid phase extraction of unbound fluoride-18. Conjugation of **38** (citrate buffer, pH 6.0, r.t., 20 min) to various fragments of the hMN-14 Anti-CEA antibody (Fab', (scFv)₂, Fab-Ad2) proved efficient (RCY 70-77%), and gave overall purified yields in the range of 49-66%.

Utilising similar methods, the radiolabelling of affibody, Z_{EGFR:1907} has been achieved [57]. This affibody has a known affinity for the epidermal growth factor receptor (EGFR) a validated target for cancer imaging. The prosthetic group **39** was prepared and conjugated to the affibody via a cysteine residue (Scheme 15). This allowed last-step radiolabelling of the **40** with Al[¹⁸F] in 15 min which gave **41** in an overall dcRCY of 15% within 40 min. This method was compared to affibody radiolabelling via the more conventional prosthetic group, **43** [73]. The synthesis of **43** was achieved by a S_N2 reaction on **42** (Scheme 16). Affibody labelling (PBS, pH 8, 60 °C, 20 min) resulted in an overall dcRCY of 41% in 2 hours. A comparison of the methods clearly displays a significant time benefit on utilising aqueous [¹⁸F]fluoride directly without an additional azeotropic drying step. On the other hand, the dcRCY was higher for the **43**-affibody conjugate (41%) than for the **41**-affibody conjugate (15%). Both probes displayed effective imaging of EGFR-expressing tumours in murine PET models.

The human epidermal growth factor HER2, has been targeted using the fluorine-18 labelled cys-affibody cys-Z_{HER2:2395} in a mouse model for ovarian cancer [74]. The reduced affibody cys-Z_{HER2:2395} was incubated with **38** to enable last-step radiolabelling. Radiolabelling was achieved using an analogous model to that described in Scheme 15, achieving an RCYs of around 25% in 30 min. The conjugate was shown to retain affinity for HER2 expressing cells *in vitro* and *in vivo* (producing PET images).

A recent comparative study contrasted four established labelling agents for the fluorine-18 labelling of the HER2-binding affibody Z_{HER2:2891} [75]. The affibody was first modified through an engineered cysteine at the C terminus to allow coupling through maleimide linkers. The prosthetic group **6** was synthesised following the protocol in Scheme 1 (RCY 38-48%) and was then conjugated to cys-Z_{HER2:2891} (aniline hydrochloride, water, 70 °C, 20 min). The total synthesis and conjugation time took 60-65 min and the overall RCY was 10-16%. The complexing agent **39** was also utilised in this study following the standard protocol in Scheme 15. The overall RCY of the radiolabelled affibody conjugate was 7-15% with a synthesis and conjugation time of 26-28 min. A further aluminium fluoride strategy was also explored using **44**. In this case synthesis and conjugation to the affibody to generate the

radiolabelled conjugate took 33 min with a RCY of 8%. A final labelling agent **45** exploited the strong silicon-fluoride ion affinity. Sufficient lability of the Si-F bond allowed aqueous fluoride-18 to be used to generate **45** by displacement of an unlabelled fluoride, as previously described (Scheme 9). Conjugation was then achieved (pH 4.0, 15 min, 95 °C) in a RCY of 36-40% in 30-35 mins.

The aluminium-fluoride method has recently been adapted to incorporate a biorthogonal inverse electron demand Diels-Alder (IEDDA) click route to antibody radiolabelling [76]. Radiolabelling of **46** with AlCl₃ and aqueous [¹⁸F]fluoride gave **47** in approximately 35 min and with dcRCY 54-65% (Scheme 17). To obtain a suitable handle for the required IEDDA conjugation, the selected antibody antiCA19.9 5B1 was incubated with an activated succinimidyl ester of *trans*-cyclooctene (TCO-NHS). The resulting TCO modified 5B1 was conjugated to the prosthetic group under ambient conditions (PBS, pH 7.4, r.t., 15 min) giving >94% RCY (Overall RCY 51-61%). The resulting radiolabelled 5B1 was shown to effectively visualise pancreatic cancer xenografts present in mice.

Recently two aluminium fluoride binding ligands have been used for radiolabelling the Z_{HER3:8698} affibody for successful imaging of HER3 positive tumours in mice [16]. Firstly, **39** was conjugated to Z_{HER3:8698} via an engineered cysteine residue with last-step radiolabelling as before (Scheme 15). The RCY was 10-27% and the radiochemistry and purification took approximately 50 min. Additionally **48** (Figure 5) was radiolabelled using the protocol in Scheme 17 with a RCY of 70-95% in 15 min. The radiolabelled ligand was then used to form the affibody conjugate with the TCO-modified Z_{HER3:8698} via an IEDDA as before. This gave an overall protein recovery of 34-55% in 70 min. By comparison the last-step **39** labelling strategy benefits from a reduced radiolabelling time of 50 min relative to the two-step **48** strategy, but this can be at the expense of yield. In the last-step labelling strategy the biomolecule is exposed to high temperatures (90-100 °C) which may result in degradation of sensitive biomolecules, thus the **48** strategy is more suitable for heat-sensitive compounds.

Work by Hendricks et al. describes a novel hybrid fluorine-18 imaging agent providing access to a PET/fluorescence molecular tomography (FMT) hybrid **50** [77]. Synthesis of **50** from **49** was achieved in a one-pot two-step process (Scheme 18) in 2 min with a total 73% RCY. Following synthesis, **50** was used to radiolabel trastuzumab – a HER2 monoclonal antibody. Radiolabelling was achieved (PBS, r.t., 15 min) in 19.9% decay-corrected RCY.

Rodriguez et al. have recently reported the use of a dioxaborolane strategy for the preparation of fluorine-18 labelled monoclonal antibodies for PET [14]. Like the aluminium fluoride methods the dioxaborolane approach avoids the less convenient [¹⁸F]fluoride drying

steps involved in the preparation of prosthetic groups. The unlabelled antibody conjugate **51** also contains a biotin handle for solid support and a hydrophilic heptacyanine optical probe. In the labelling step **51** was immobilised on a solid-phase support and exposed to aqueous [^{18}F]fluoride. The resulting fluorolysis cleaved the conjugate from the solid support, releasing the radiolabelled antibody conjugate **52** and the remaining biotinylated **53** (Scheme 19). The inclusion of the optical probe allows for a PET-near infrared fluorescence (NIRF) multimodal imaging strategy.

The use of [^{18}F]FDG **2** as an imaging agent for PET is well established due to its particular ability to localise in a range of cancer cell types *in vivo* and it is the most widely used tool in PET diagnosis [78]. Consequently, **2** is available in most clinical radiochemical environments specialising in PET and this makes it an ideal starting point for prosthetic group syntheses for the labelling of biomolecules. Rashidian et al. have reported the synthesis of the VHH conjugate **58** [79]. Firstly, the reaction of **2** with **54** trapped the open chain **55**. Meanwhile, sortase substrate **56** was exposed to sortase and the nanobody preconjugate VHH-LPETG-XX and the resulting biotransformation gave the TCO-nanobody conjugate **57**. A rapid click reaction between **55** and **57** gave the radiolabelled antibody **58** (Scheme 20). The total synthesis time from **2** was 25 mins and RCY were in excess of 25%. The labelled antibody conjugate was used to successfully detect small heterotopic pancreatic tumour transplants in mice by PET imaging.

Further work from Rashidian et al. has demonstrated that the substrate scope for the sortase-mediated process facilitates modular assembly (Scheme 21) [80]. Firstly, **1** was elaborated to monomeric VHH conjugate **61**. This was achieved by first amide coupling **1** with **59** to give **60**. Meanwhile, the sortase product **57** was prepared as previously (Scheme 20). The click reaction between **57** and **60** gave the conjugate **61**.

Alternatively, **60** underwent a 'click' reaction with the bifunctional (azide and TCO-containing) sortase product **62** followed by exposure to DBCO-modified VHH, to affect a second 'click' reaction. This gave the dimer **63** which features two VHH nanobodies. Dimers such as **63** were shown to have different avidity and kinetic profiles to monomers such as **61**. The modularity of the protocol (Scheme 21) provides access to an array of conjugates. For example: **62** can be replaced with sortase products featuring other click handles or a PEG chain for solubility. Variability of these constructs was shown to have a significant effect on *in vivo* properties such as avidity, uptake and clearance profiles when targeting a range of targets [80].

The use of enzymes for the radiolabelling of biomolecules related to PET is a new and relatively unexplored field. Recently lipoic acid ligase was shown to be a suitable reagent for the site-specific radiofluorination of biomolecules [17]. The octanoate **65** was prepared synthetically (3h, dcRCY 26 %) and was enzymatically coupled to Fab fragment **64** to give the radiolabelled amide conjugate **66** (Scheme 22). Notably, site specificity was achieved by introducing a LAP peptide tag into the Fab by standard cloning techniques. Enzyme incubation time was relatively short (10-15 min) and conjugation yields were high (~95%) with low loading of the Fab protein precursor (10 nmol). The lipoic acid ligase (and enzyme-mediated transformations in general) has the benefit of utilising mild conditions which are unlikely to denature the protein biomolecules.

3. Conclusion

This review has summarised the efforts and ongoing status of radiolabelling antibodies with the fluorine-18 isotope, for positron emission tomography applications. The radiolabelling of antibodies and their fragments with fluorine-18 continues to receive ever-growing attention [81–83]. The account highlights the current activity and challenges in the field. It has tried to identify the different approaches taken using fluorine-18 prosthetic groups, combining these with low molecular weight antibody fragments and affibodies to match clearance rates with the half-life of the fluorine-18 isotope. This has required ingenuity in both isotope tagging strategies and antibody fragment engineering. Last step labelling from aqueous [¹⁸F]fluoride, through macrocycle secured aluminium is emerging as a popular and practical method. Further methods using aqueous [¹⁸F]fluoride are also gaining prevalence. High-yielding biorthogonal click reactions can provide modular platforms for conjugates with tuneable avidity and kinetics, and mild enzymatic methods are likely to garner further attention in the future. Efforts to label full sized antibodies with fluorine-18 remains extremely challenging. This generally requires pre-targeting strategies, where the antibody is administered several days in advance of a low molecular weight [¹⁸F]-labelled tracer which finds its way to, and binds or reacts with the pre-localised antibody *in vivo*. Significant challenges remain which will benefit from additional innovative solutions.

4. References

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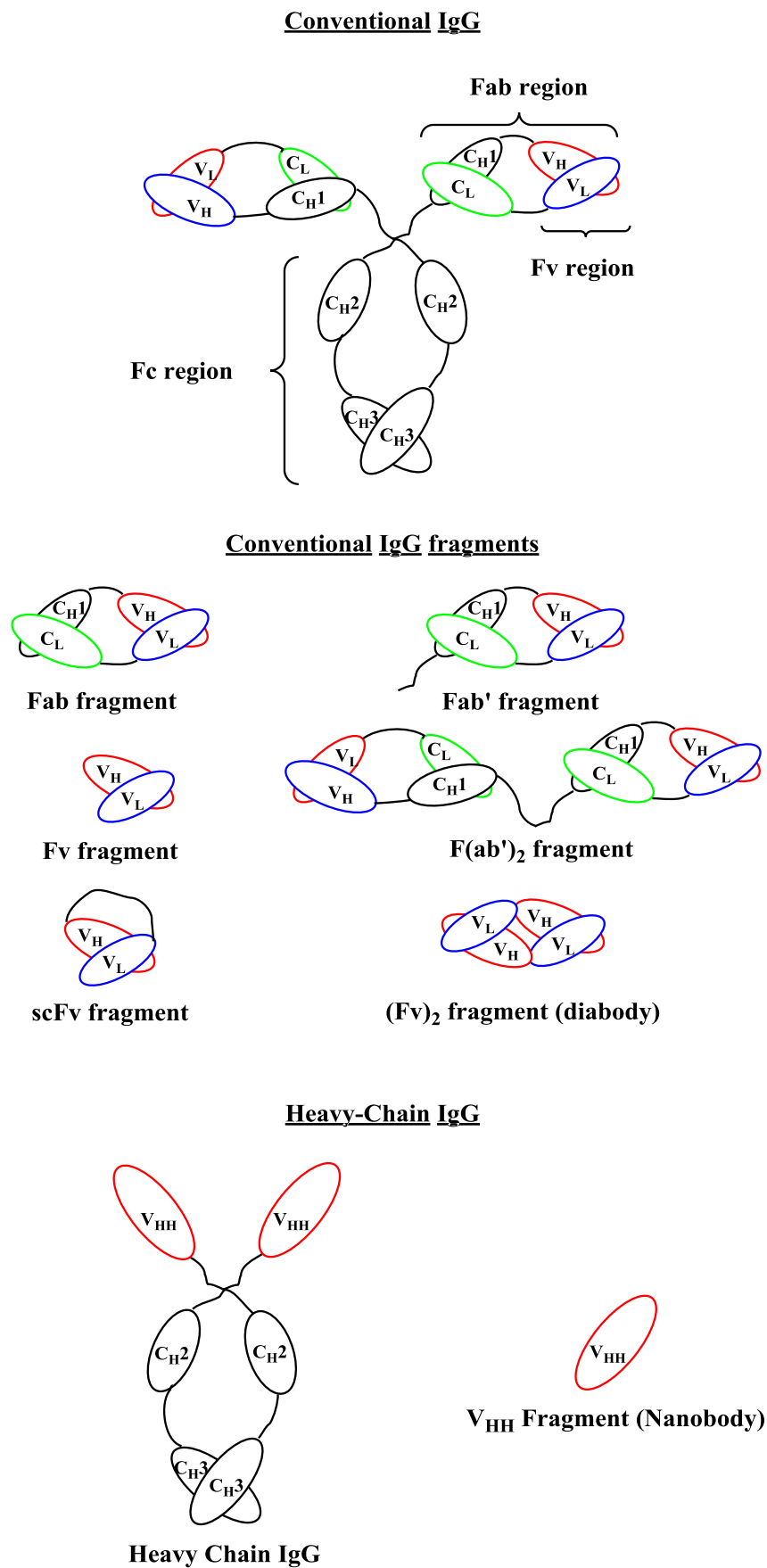


Fig. 1. Conventional and Heavy-Chain IgG and their fragments, adapted from Joosten et al. [20].

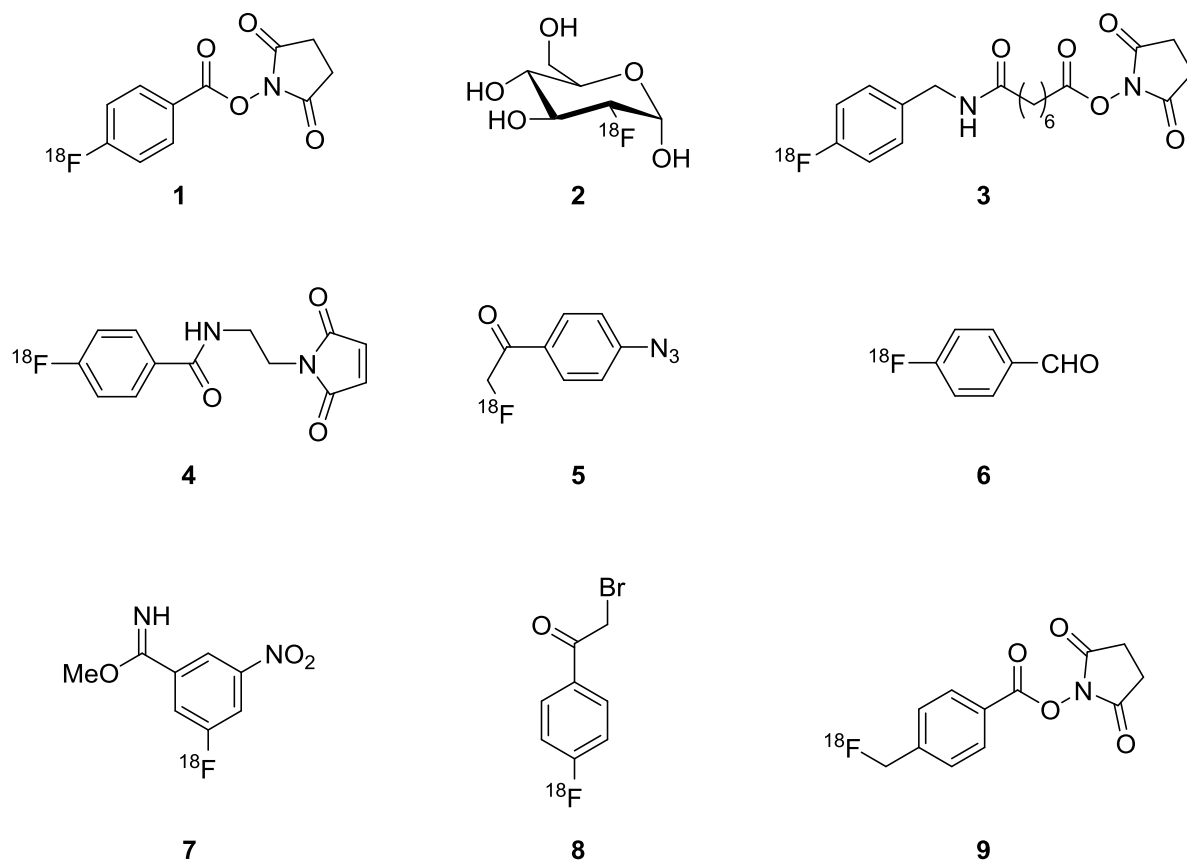


Fig.2. Compounds initially used for fluorine-18 radiolabelling of antibodies and their fragments.

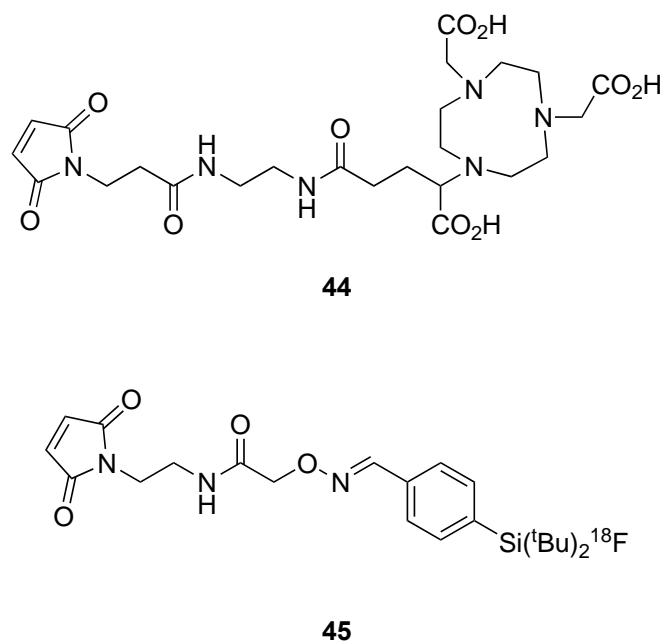


Fig. 3. Structure of 44 and 45 [75].

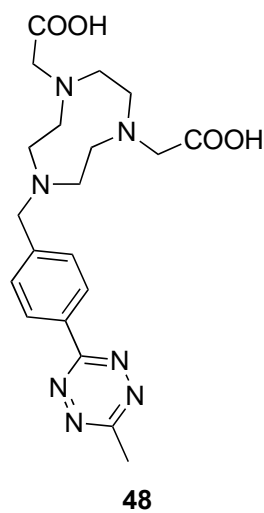
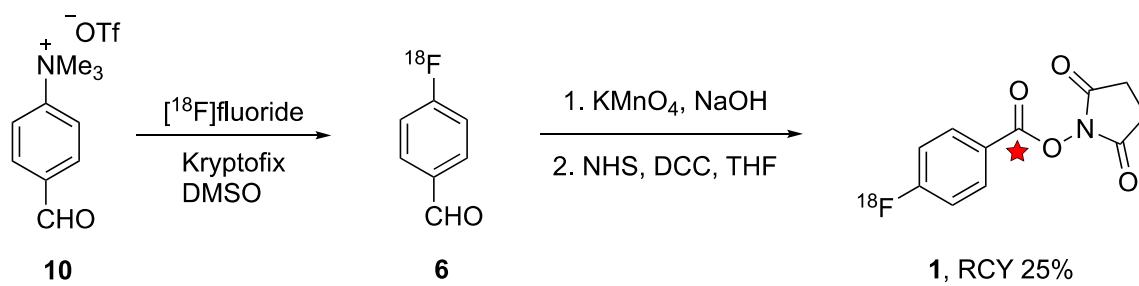
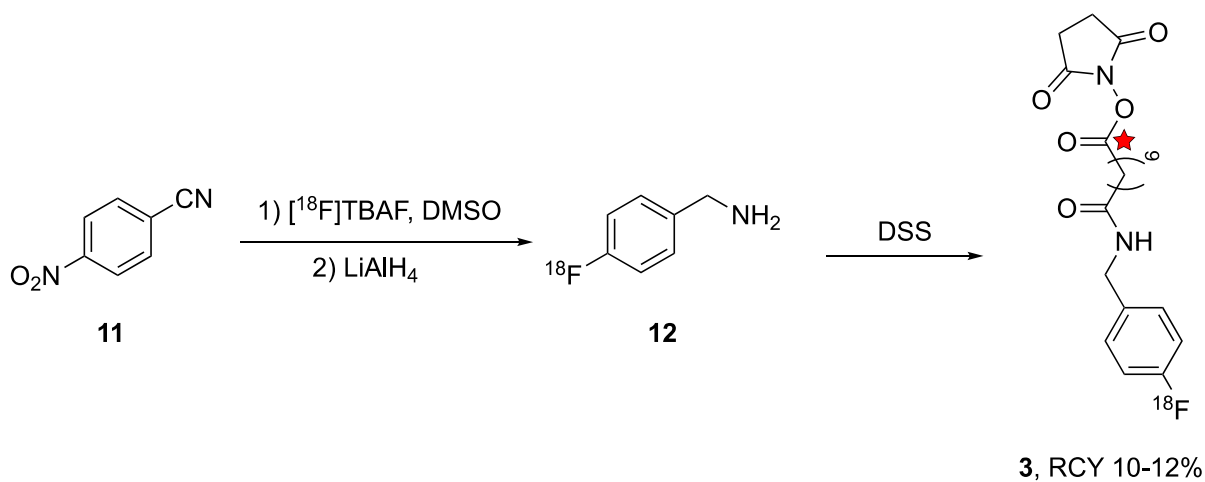


Fig. 4. Structure of **48** [16].



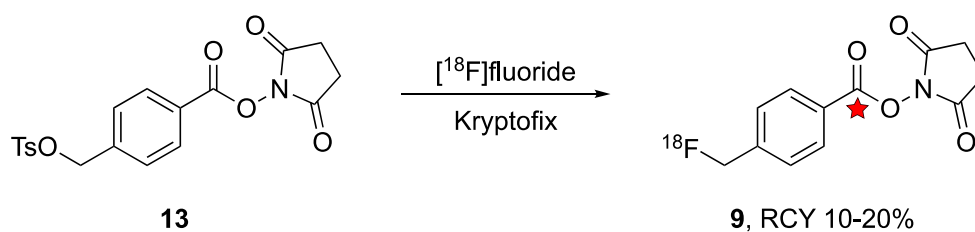
★ Site of lysine residue conjugation

Scheme 1. Radiosynthesis of **1** [13,27,28].



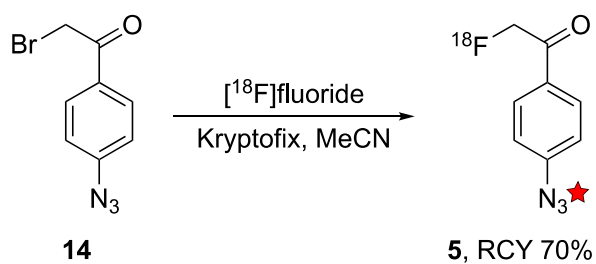
★ Site of lysine residue conjugation

Scheme 2. Radiosynthesis of **3** [30–34].



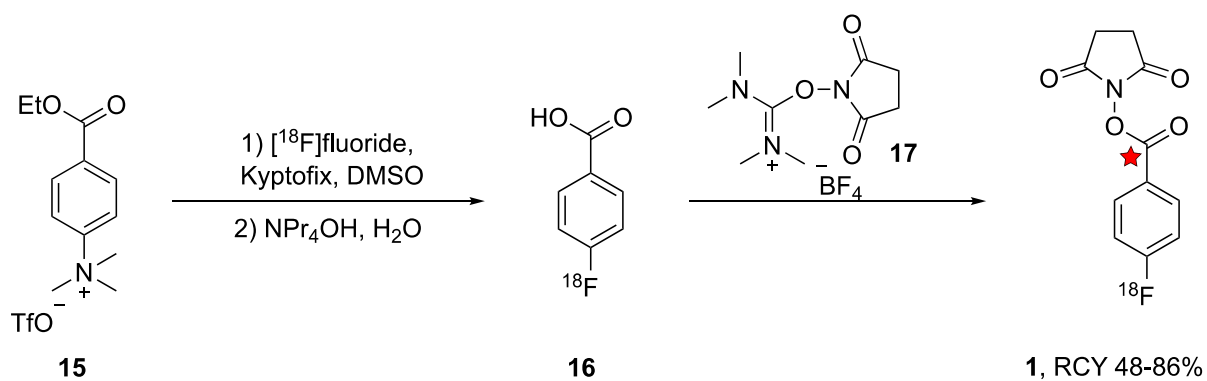
★ Site of lysine residue conjugation

Scheme 3. Radiosynthesis of **9** [35,36].



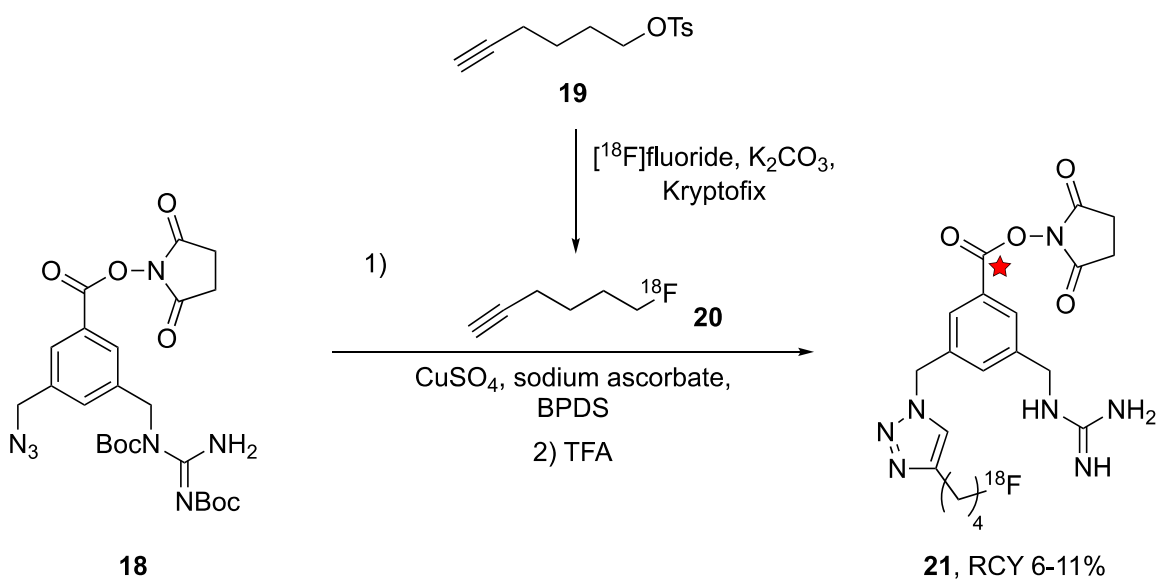
★ Site of photoactivation for affinity labelling

Scheme 4. Radiosynthesis of **5** [12].



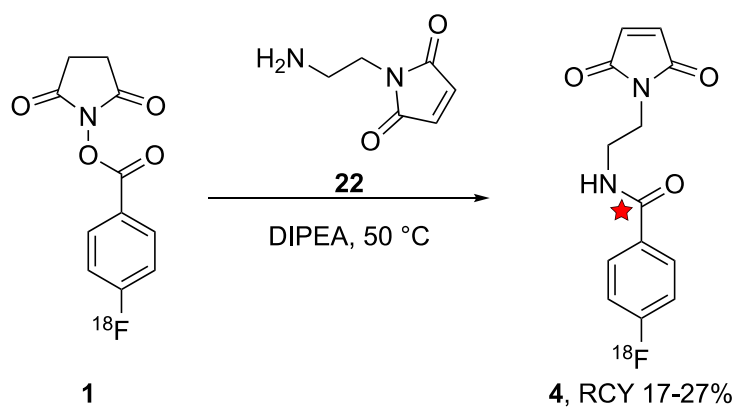
★ Site of lysine residue conjugation

Scheme 5. Modified radiosynthesis of **1** [39].



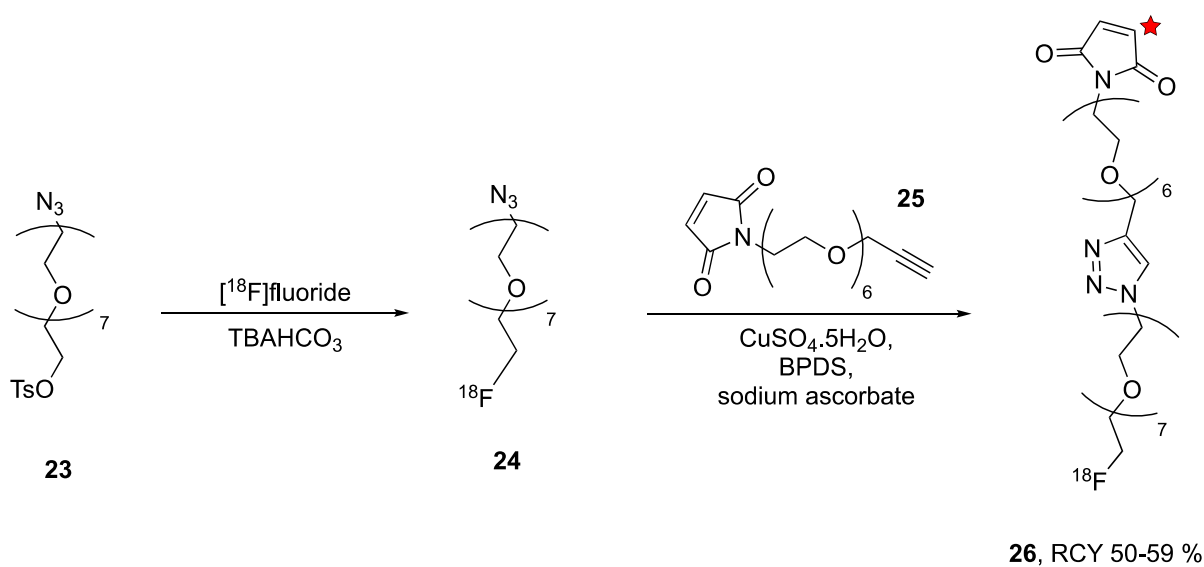
★ Site of lysine residue conjugation

Scheme 6. Radiosynthesis of **21** [53].



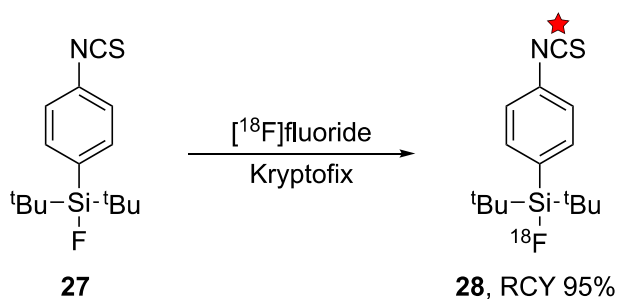
★ Site of lysine residue conjugation

Scheme 7. Synthesis of **4** from **1** [56].



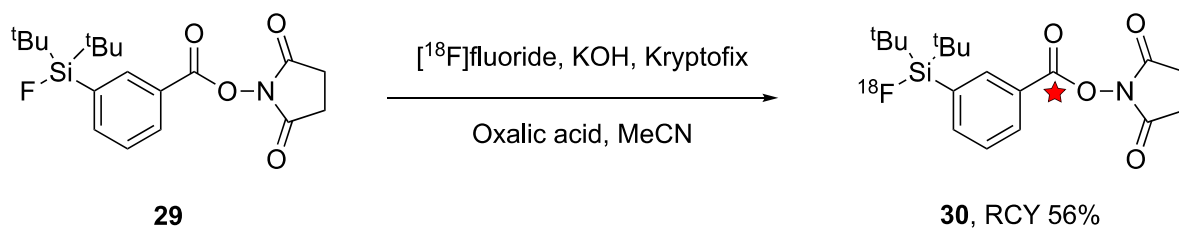
★ Site of cysteine residue conjugation

Scheme 8. Radiosynthesis of **26** [65].



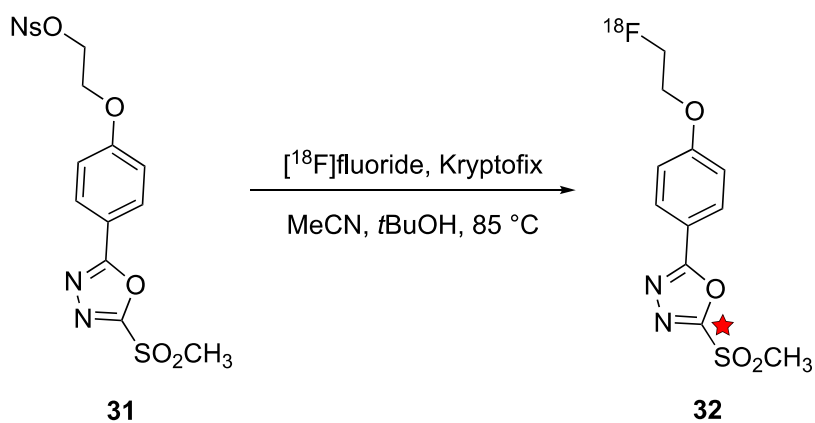
★ Site of lysine residue conjugation

Scheme 9. Radiosynthesis of **28** [66].



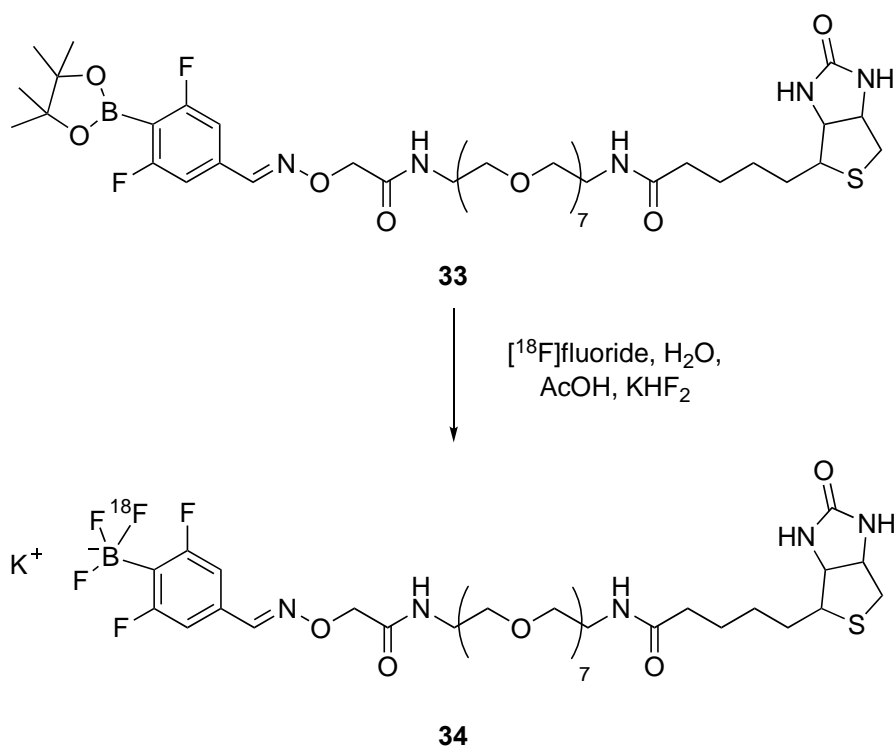
★ Site of lysine residue conjugation

Scheme 10. Radiosynthesis of **30** [67].

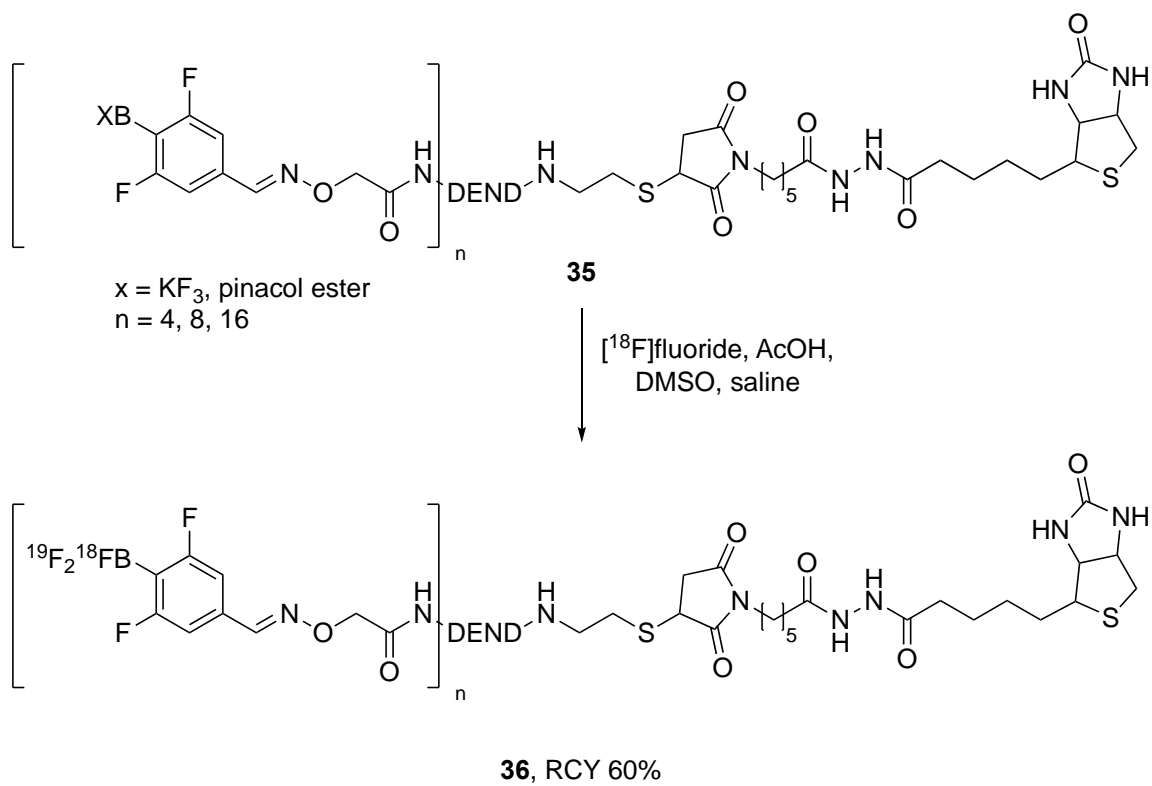


★ Site of cysteine residue conjugation

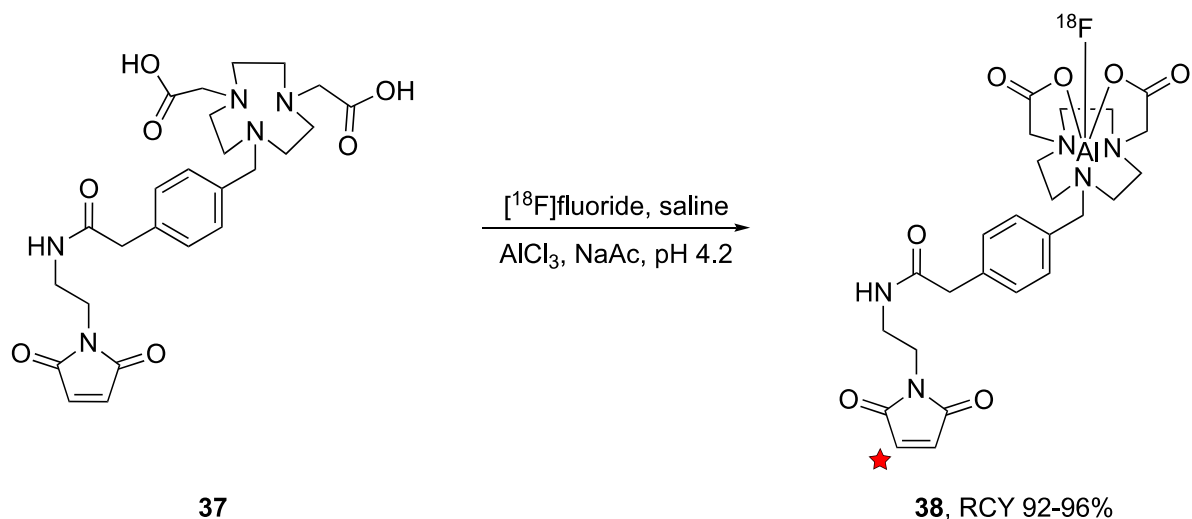
Scheme 11. Radiosynthesis of **32** [70].



Scheme 12. Radiosynthesis of **34** [18].

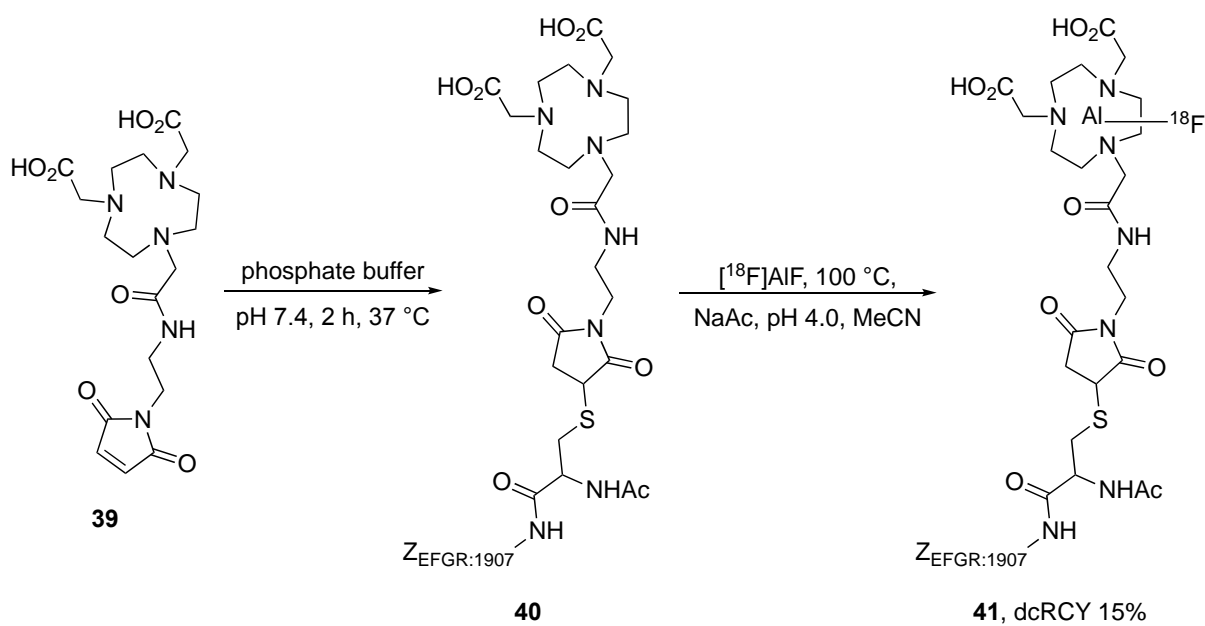


Scheme 13. Radiosynthesis of **36** [71].

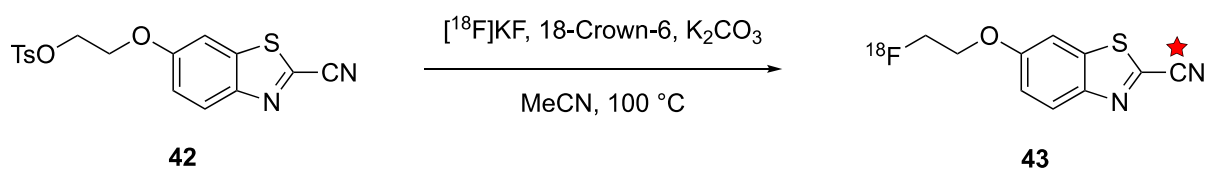


★ Site of cysteine residue conjugation

Scheme 14. Radiosynthesis of **38** [15,72].

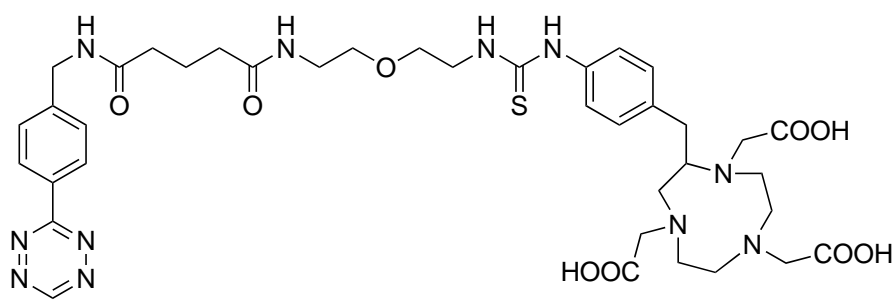


Scheme 15. Conjugation and radiolabelling of **39** [57].

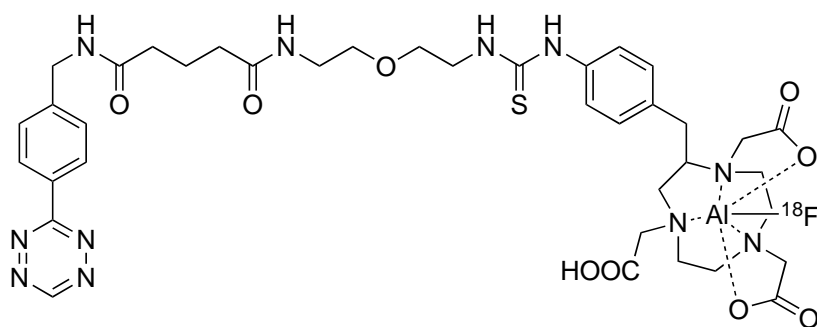
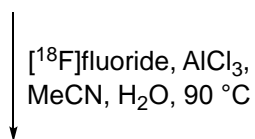


★ Site of cysteine residue conjugation

Scheme 16. Radiosynthesis of **43** [73].

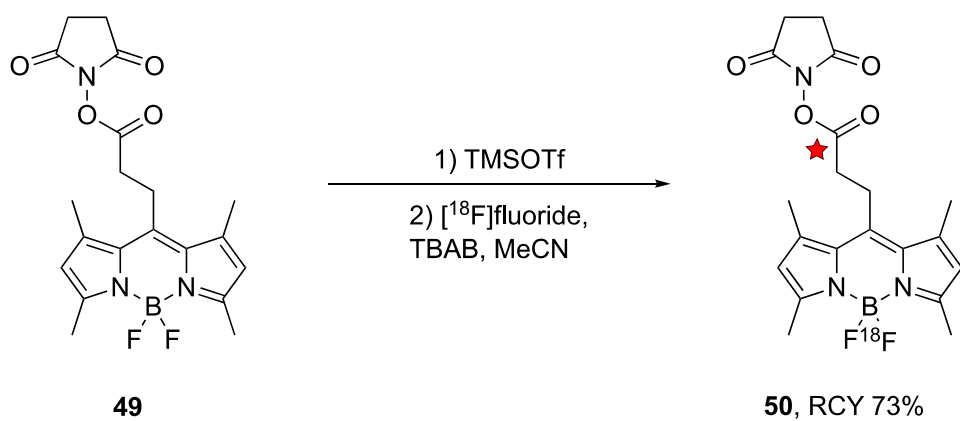


46



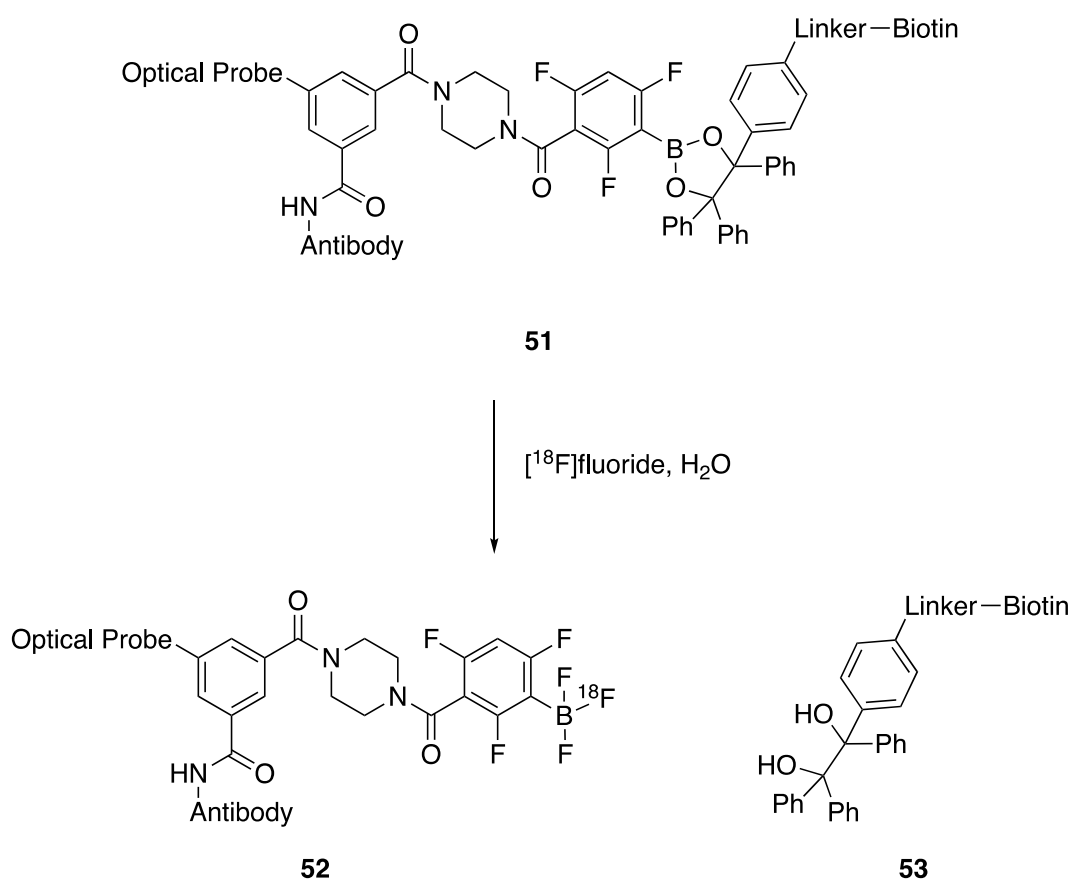
47, dcRCY 54-65%

Scheme 17. Radiosynthesis of 47 [76].

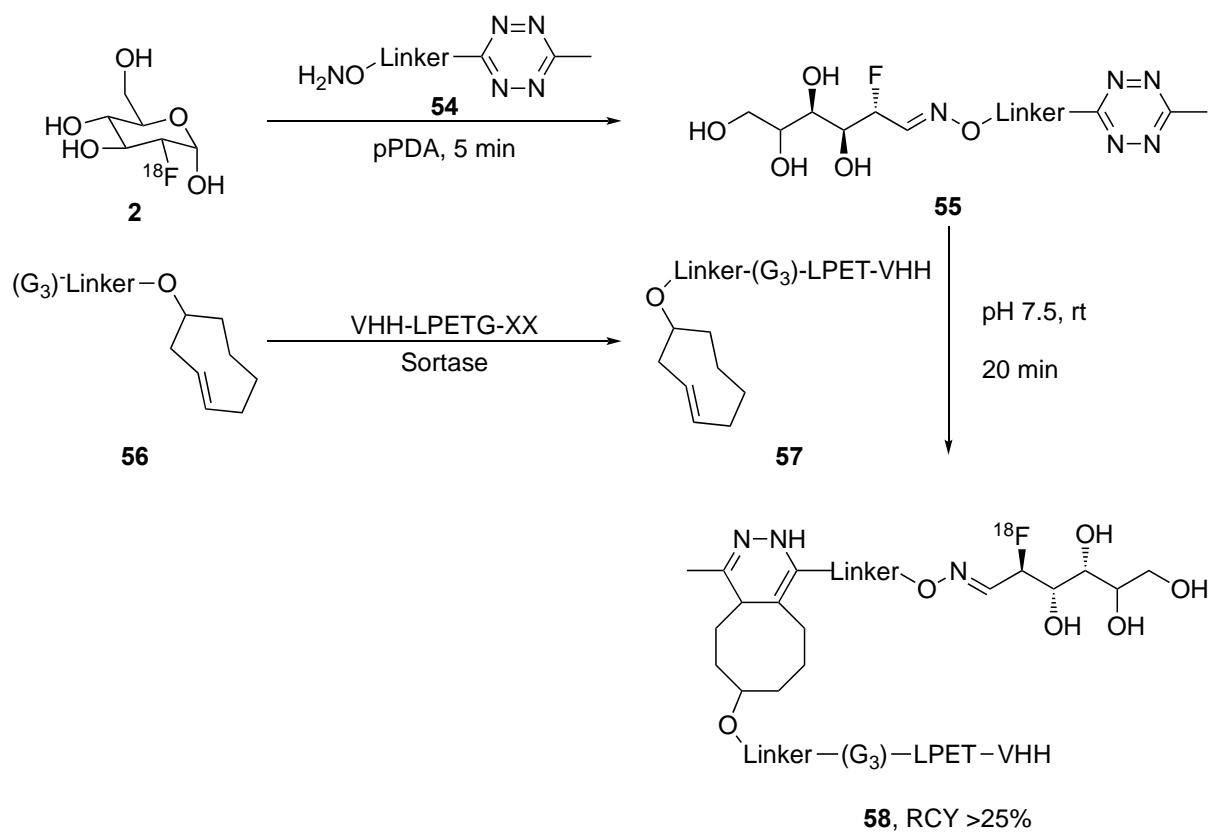


★ Site of lysine residue conjugation

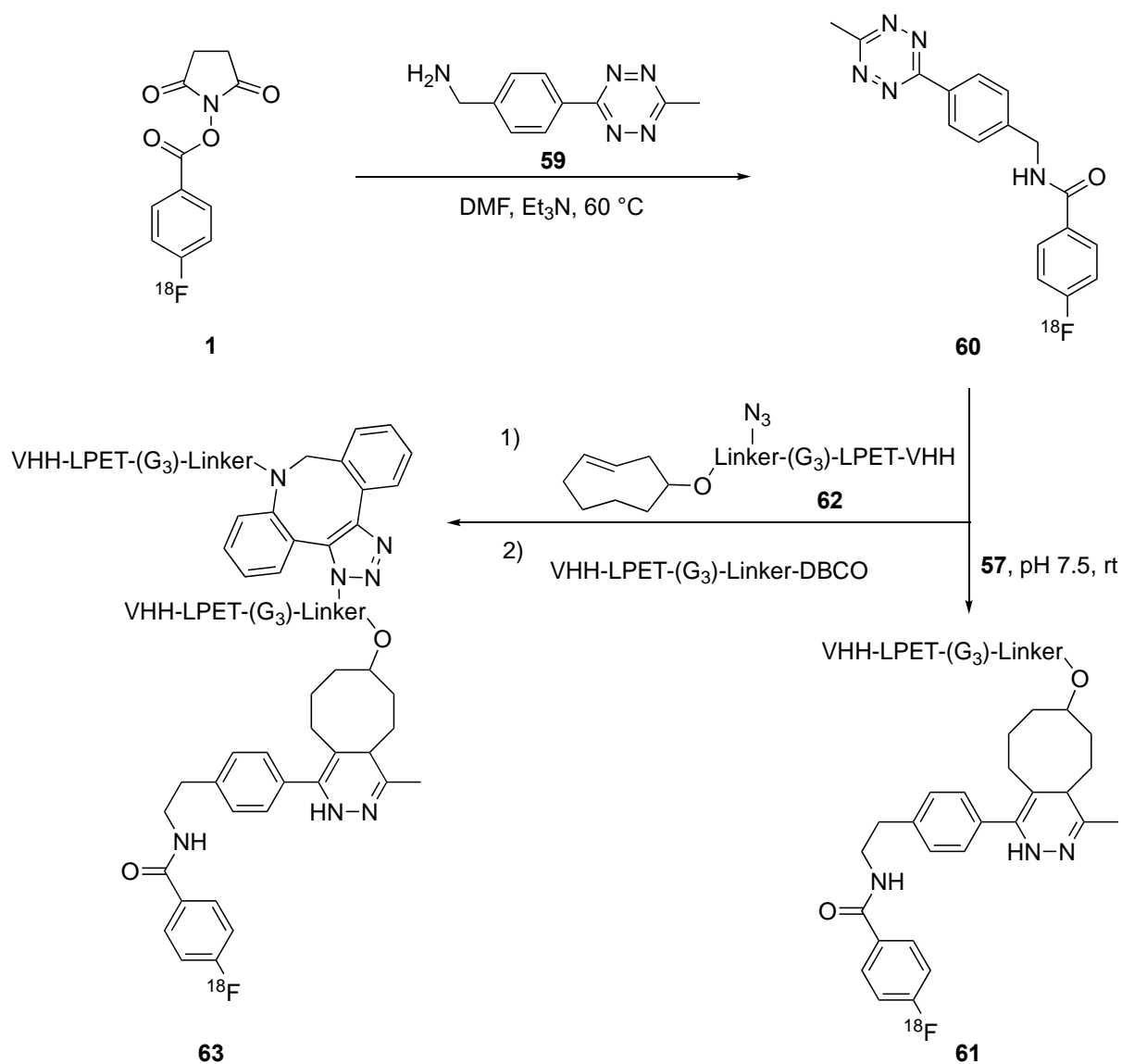
Scheme 18. Radiosynthesis of **50** [77].



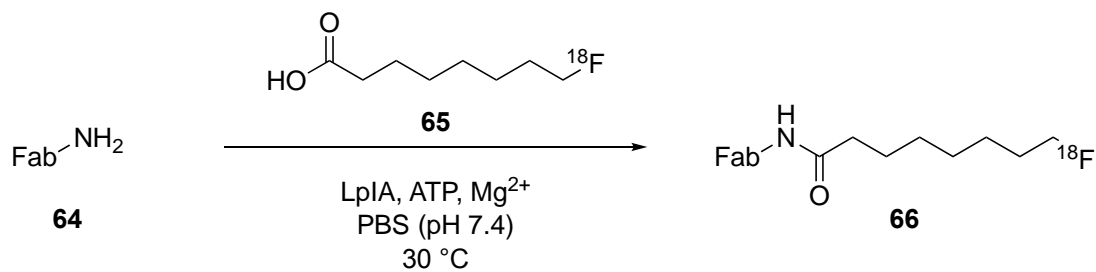
Scheme 189. Radiolabelling of **51** to give **52** [14].



Scheme 20. Elaboration of **2** to **58** via enzymatic and click processes [79].



Scheme 21. Use of modular click reactions to radiolabel nanobody monomer **61** and dimer **63** [80].



Scheme 22. Enzymatic radiolabelling of Fab fragment **64** by lipoyl-CoA ligase-mediated amidation [17].