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Chemoselective ^{18}F-incorporation into pyridyl acyltrifluoroborates for rapid radiolabelling of peptides and proteins at room temperature†

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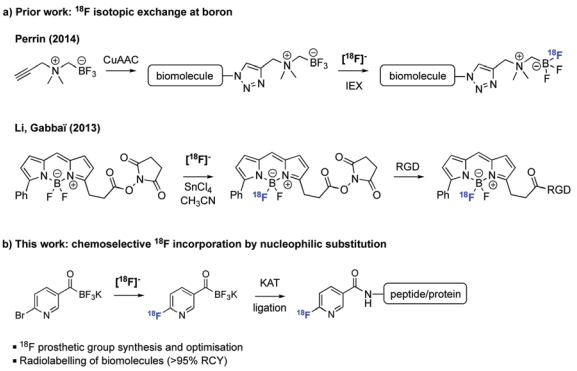
A new prosthetic group is reported for ^{18}F-labelling of peptides and proteins based on the chemoselective ligation of potassium acyltrifluoroborates (KATs) and hydroxylamines without any detectable ^{18}F/^{19}F isotope exchange at the acyltrifluoroborate moiety. The new building block is appended *via* a common amide bond at room temperature with no need for protecting groups which enables an effective orthogonal ^{18}F-radiolabelling.

Positron emission tomography (PET) can visualise, characterise and quantify biological processes at the cellular and molecular levels *in vivo* non-invasively, with high sensitivity and spatial resolution. It has evolved into a powerful imaging tool, contributing decisively in both basic research and medical decision-making.[1] Fluorine-18 is the most frequently utilised radionuclide for diagnostic PET imaging since its decay properties (^{18}F; β^+ 0.635 MeV, 97% abundance, $t_{1/2}$ = 109.8 min) provide significant advantages over other PET radionuclides.[2] The ever-increasing use of biomolecular vector-based imaging agents has brought forth a high demand for ^{18}F-radiosynthesis strategies that are fast, mild and selective. However, the harsh conditions required for direct ^{18}F-labelling (high temperatures, basic pH, dry organic solvents), the extremely low concentration levels of ^{18}F and its relatively fast radioactive decay make the development of ^{18}F radiopharmaceuticals of complex molecules a challenging task.

Prosthetic groups have expanded the field of radioactive bioconjugation in recent years (Scheme 1).[3] These are small bifunctional molecules that can be radiolabelled with a radionuclide and subsequently appended to biomolecules. The great majority of prosthetic groups have relied upon reactions with natural amino acids (most notably couplings between activated carboxyl groups and lysines and Michael additions between maleimides and cysteines).[6–9] However, the complete loss of regiochemical control during the labelling of a biomolecule when more than one (unprotected) lysine or cysteine are present requiring time-consuming protection and/or purification steps prompted radiochemists to shift their attention towards ligations of higher specificity. As such, a wide panel of chemoselective ligation reactions have been used in PET radioligand development.[10] Lately, the oxime-ligation methodology has gained increased attention.[11]



Scheme 1 Overview of work on boron-based ^{18}F prosthetic groups.[4,5]

The fairly recent discovery of (almost) ideal orthogonal ligations gave a new impetus in the development of advantageous ^{18}F-labelling prosthetic groups. Among these reactions, the Cu-catalyzed azide–alkyne cycloaddition (CuAAC),[12] the strain-promoted azide–alkyne cycloaddition (SPAAC, azides-cyclooctenes)[13] and the inverse electron-demand Diels–Alder reaction (IEDDA)[14] are the most prevalent. The selectivity, ease and modularity of these ligations make them well-suited for the construction of complex ^{18}F-labelled tracers. But despite their utility, these reactions exhibit several flaws that limit their universal application (*e.g.*, use of toxic additives for CuAAC and formation of bulky hydrophobic linkages for SPAAC and IEDDA).[15] Therefore novel approaches to

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facilitate ^{18}F-labelling and diversify the pre-existing library of ^{18}F-radiotracers are still needed.[16]

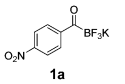
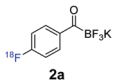
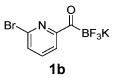
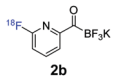
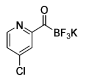
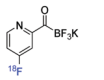
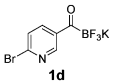
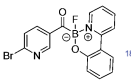
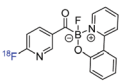
In 2012, Bode and Molander showed that the reaction between potassium acyltrifluoroborates (KATs) and various hydroxylamines leads to the formation of amide bonds under aqueous conditions at room temperature with a second-order rate constant of 20 M^{-1} s^{-1}.[17] The reaction shows excellent chemoselectivity and tolerates all common unprotected functional groups typically found on proteins and peptides. KAT ligation is faster than the majority of the ligations in common use and it has the distinct advantage of using chemically stable, easily handled functional groups.[18] The Bode group successfully used this method for the modification of a synthetic GLP1 analogue[19] and for the two-step modification of recombinant proteins.[20] In order to harness the advantages of the KAT ligation for radiochemistry applications, we sought to develop a new ^{18}F-labelling prosthetic group based on this chemistry.

Despite the advantages of KATs for rapid and chemoselective ligation, installation of the ^{18}F could be complicated by ^{18}F/^{19}F isotopic exchange at the trifluoroborate group which would inevitably lower the overall ^{18}F-incorporation yield. There is already an impressive body of work on ^{18}F exchange with organotrifluoroborates. Most notably, Perrin and co-workers have employed ^{18}F/^{19}F isotopic exchange on trifluoroborates for PET imaging.[21,22] Their studies showed that alkyl acyltrifluoroborates lose a fluoride with a long solvolysis half-life of >2000 min.[23] We hypothesised that [^{18}F]fluoride ions would favour nucleophilic aromatic substitution over ^{18}F/^{19}F isotopic exchange under non-aqueous solvents at higher pH, as these isotopic exchange reactions are usually carried out under aqueous acidic conditions or with strong Lewis acids.[6,24,25]

For the purpose of this study, five precursors were evaluated for ^{18}F-incorporation (Table 1): KAT derivatives **1a–d** and monofluoroacylboronate **1e**. For the substrate scope, we selected targets likely to be metabolically stable and not prone to defluorination, in contrast to alkyl fluorides.[26,27] Defluorination is particularly undesired in the context of *in vivo* PET imaging, due to bone uptake of free [^{18}F]fluoride.

All precursors for the radiolabelling (Table 1, **1a–e**) and their respective ^{19}F-reference compounds were synthesised according to the protocols developed by the Bode group (see ESI†). The precursors were subjected to typical conditions for nucleophilic aromatic substitution – [^{18}F]fluoride ions were eluted with a solution of Kryptofix®2.2.2/Cs_2CO_3 from an anion exchange cartridge then azeotropically dried with CH_3CN. Precursors **1a–e** in DMSO were added and the reaction mixture was heated at 150 °C for 10 min. ^{18}F-Incorporation yield and product identity were determined by UPLC (see ESI†) and HPLC respectively, equipped with a radio-detector. KAT **1a** with the electron-withdrawing nitro leaving group failed to afford the desired labelled compound since the electron-withdrawing effect of the KAT was not sufficient to activate the aromatic ring. Both precursors **1b** and **1c** bearing the KAT group in *meta*-position to the halogen showed less than 5% ^{18}F-incorporation (entries 2 and 3). Precursor **1d** afforded product **2d** with 23.6% ^{18}F-incorporation (entry 4), confirming a synergistic effect of the two activating groups. Acylmonofluoroboronate **1e** gave product **2e** with 12% yield (entry 5).

Table 1 Results of the ^{18}F-incorporation yields with and without DABCO

Entry	Precursor	Labelled prosthetic group	^{8}F-Incorporation without DABCO[a]	^{18}F-Incorporation with DABCO[a] (140 mM)
1	 1a	 2a	0%	n/a
2	 1b	 2b	3.5% ± 2.5, n = 3	35.5% ± 6.8, n = 5
3	 1c	 2c	2.3% ± 1.1, n = 3	3.2% ± 0.7, n = 3
4	 1d	 2d	23.6% ± 1.1, n = 3	44.7% ± 6.2, n = 4
5	 1e	 2e	12.0% ± 3.1, n = 3	n/a

[a] 10 μmol **1a–1e**, [^{18}F]CsF-$K_{2.2.2}$, dry DMSO (300 μL), 150 °C, 10 min; n = number of trials.

During the course of our experiments, Pike *et al.* reported the enhancing effects of 1,4-diazabicyclo[2.2.2]octane (DABCO) on ^{18}F-radiolabelling of substituted 2-halopyridines.[28] As anticipated, the addition of DABCO (140 mM) to the reaction mixture of the chloro-precursor **1c** only marginally improved the ^{18}F-incorporation (entry 3), as this method is effective only for 2-halopyridines. In contrast, a remarkable improvement was observed for precursor **1b** and **1d** (entries 2 and 4), with product **2d** exhibiting the highest ^{18}F-incorporation of 44.7%. In light of these findings, **1d** became the precursor for our new prosthetic group **2d**, termed [^{18}F]FPAT (6-fluoropyridyl acyltrifluoroborate).

Subsequently, further optimisation experiments were performed, the results of which are summarized in the ESI.† We first established that the same high radiochemical conversions could be obtained at 120 °C with a lower amount of the precursor (1 mg, 3.4 μmol). Furthermore, aqueous DMSO (0.1–2% H_2O v/v) had a positive impact on ^{18}F-incorporation. Most importantly, the yield could be enhanced by avoiding the conventional azeotropic drying step. We were delighted to see that these optimisations significantly improved the conversion to 71.1% ± 12.5 (n = 11). This increase in yield can be attributed to less adsorption and higher availability of [^{18}F]fluoride in the reaction medium. Moreover, the tolerance towards H_2O could be rationalised by the strong charge interactions between the free [^{18}F]fluoride ion and the positively charged quaternary nitrogen of DABCO at the expense of weaker interactions with water. Employing these conditions with NBu_4HCO_3 instead of Cs_2CO_3 further improved the radiochemical conversion to 90.5% ± 5.2 (n = 6). However, this caused broadening of the peaks and poor separation of [^{18}F]FPAT during the HPLC purification step.

Exchanging the base to K_2CO_3 afforded [^{18}F]FPAT with 84.2% ± 1.2 (n = 5) ^{18}F-incorporation while maintaining a clean HPLC purification profile. This procedure was deemed optimal for [^{18}F]FPAT, affording the prosthetic group in good molar activities of 81 ± 26 (n = 7) GBq μmol^{-1} and isolated RCYs of 58% ± 2.5 (decay

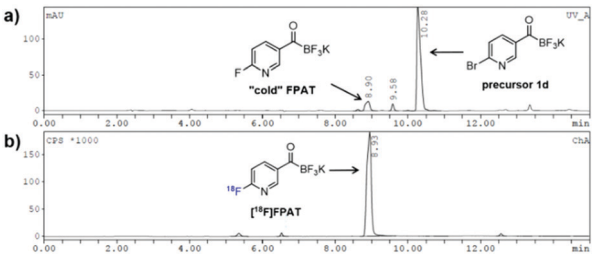


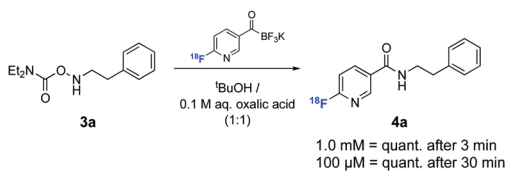
Fig. 1 ^{18}F-Labelling of bromo-precursor **1d**. (a) UV-HPLC chromatogram; (b) γ-HPLC chromatogram (crude).

corrected, $n = 3$). Unlike similarly structured ^{18}F-nicotinamide based prosthetic groups (*e.g.*, [^{18}F]FPy-TFP)[29] and other amine reactive building blocks (*e.g.*, ^{18}F-SFB),[30] which all non-selectively react with Lys residues, [^{18}F]FPAT reacts chemoselectively with hydroxylamines without the need for protecting groups.

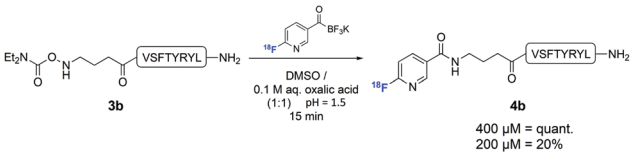
We were also curious to see whether the acyltrifluoroborate engages in ^{18}F/^{19}F isotopic exchange. There was no observable radiosignal corresponding to the bromo-precursor **1d** (10.3 min) on the HPLC after ^{18}F-incorporation (Fig. 1). The high ^{18}F-incorporation yields indicated that overall there was a strong preference for nucleophilic substitution over isotopic exchange under the radiolabelling conditions. The origin of the remarkably slow exchange of the fluorides on the KAT moiety is under further investigation.

We next evaluated the ligation efficiency of [^{18}F]FPAT at ambient temperature with various hydroxylamine substrates **3a–c** (results summarised in the ESI†). For all succeeding experiments, [^{18}F]FPAT was purified to avoid competing reactions with excess remaining precursor. These ligations were performed in aqueous medium with organic solvents (^{t}BuOH or DMSO) as additives for solubilising the substrates. Aqueous oxalic acid was also added since KAT ligations are faster under acidic conditions.[19] Complete conversion of [^{18}F]FPAT to amide **4a** was observed within 3 min at 10 mM and 1 mM concentrations of the precursor. Decreasing the concentration to 0.1 mM led to 87% conversion in 15 min and full conversion after 30 min (Scheme 2).

In order to establish that this method could be used to introduce ^{18}F into biologically relevant molecules, we selected a short eight-residue peptide. The peptide VSPTYRYL was synthesised by standard Fmoc-SPPS on a Rink Amide resin, and the *N*,*N*-diethylcarbamoylhydroxylamine functional group introduced at the N-terminus. ^{18}F-Labelling of **3b** was carried out with [^{18}F]FPAT (1–4 GBq) using peptide concentrations typically reported for similar substrates[31] (Scheme 3). At 800 μM and 400 μM, the ligation afforded the labelled peptide **4b** quantitatively after only 15 min. Reducing the concentration to 200 μM resulted in 20% incorporation after 15 min.



Scheme 2 Radiolabelling of hydroxylamine **3a** with [^{18}F]FPAT. Yields determined by γ-UPLC.

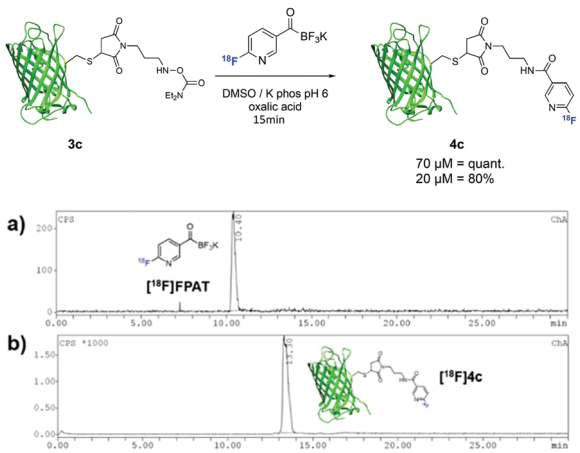


Scheme 3 Radiolabelling of hydroxylamine peptide **3b** with [^{18}F]FPAT. Yields determined by γ-HPLC.

To further extend this method towards larger biomolecules, we modified sfGFP(S147C)[20] with a maleimide functionality bearing the hydroxylamine group at the exposed cysteine residue (Scheme 4). Due to the low concentrations of the protein used, small amounts of [^{18}F]FPAT (50–100 MBq) were used respectively to avoid interference of the cold [^{19}F]FPAT. At 70 μM, the reaction successfully afforded the labelled protein **4c** in quantitative yields, while a three-fold lower concentration led to 80% conversion to the conjugation product.

In order to confirm that [^{18}F]FPAT is resistant towards defluorination *in vivo*, which is particularly important for applications in pretargeting, [^{18}F]FPAT was injected into a C57BL/6 mouse for a pilot PET/CT study (Fig. 2). No bone uptake was observed over the 90 minute time period, establishing that no defluorination of the prosthetic group was evident, indicating the high stability of the [^{18}F]fluoride incorporated into this scaffold.

In summary, we developed a new radioconjugation strategy for the ^{18}F-radiolabelling of peptides and proteins based on KAT ligation. The novel prosthetic group [^{18}F]FPAT was prepared in high radiochemical yield and good molar activity without any detectable ^{18}F/^{19}F isotopic exchange. [^{18}F]FPAT coupled selectively within minutes in aqueous medium with *O*-diethylcarbamoylhydroxylamines at low concentrations with excellent conversions. Compared to modern orthogonal ^{18}F-ligation approaches (*e.g.*, SPAAC, IEDDA) where building blocks are appended to the biomolecules with bulky hydrophobic linkages, [^{18}F]FPAT is tethered *via* a robust and innocuous amide bond which is expected to constitute a minimal perturbation of the native biomolecule. Moreover, compared to the increasingly used oxime ligation methodology,



Scheme 4 Radiolabelling of protein sfGFP(S147C) **3c** with [^{18}F]FPAT; (a) γ-HPLC chromatogram of [^{18}F]FPAT; (b) γ-HPLC chromatogram of ligation (crude).

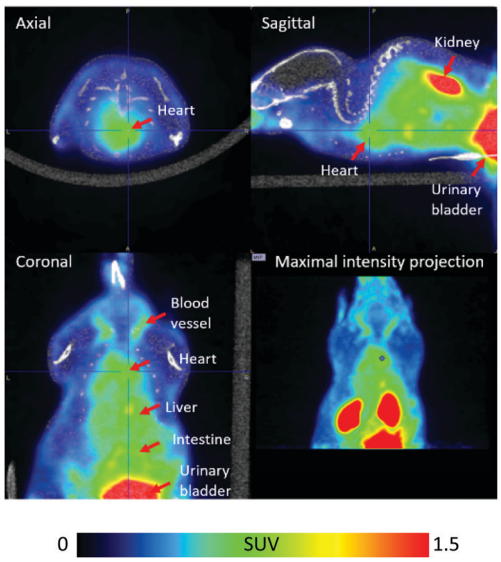


Fig. 2 PET/CT images of [^{18}F]FPAT averaged from 1 to 91 min post-tracer injection. The colour bar designates the standardised uptake value (SUV).

[^{18}F]FPAT appears advantageous since its conjugation with hydroxylamine-functionalized biomolecules is unconditionally fast, and the amide adducts are expected to possess increased stability over a wide pH range in contrast to oxime conjugation products. Therefore, this approach meets all criteria of an effective orthogonal radiolabelling strategy and has the potential to become a method of choice for the ^{18}F-radiolabelling of biomolecules. Implementation of this novel radiolabelling strategy to peptides and proteins of biological interest is currently under investigation, as well as for applications in pretargeting.

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Conflicts of interest

There are no conflicts to declare.

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