

Automated synthesis of ^{18}F radiolabelled indole containing Oncrasin-like molecules; a comparison of iodonium salts and boronic ester chemistry.

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Abstract

Oncrasin-1 is a small molecule which was identified from a screen of KRAS mutant cancer cells and has shown specificity for KRAS mutant cell killing. We aimed to develop a radiolabelled form of Oncrasin-1 to enable in-vivo imaging of mutant KRAS expression in malignant tumours. This work outlines the synthesis of 3 fluorinated derivatives and development of iodonium salt and boronic ester precursors for radiolabelling with the ^{18}F isotope. Attempted synthesis of iodonium salts showed that the indole structure is incompatible with oxidizing conditions required for iodonium salt formation. Synthesis and radiolabelling of boronic acid pinacol ester precursors were successful and resulted in RCY of 10–25% across the 3 different substitution patterns. The successful synthesis of these tracers will allow for investigation of Oncrasin based molecules as potential diagnostics for cancers expressing mutant KRAS protein.

Background

Human RAS genes have been identified as one of the most frequently mutated oncogene family in all cancers, with estimations of up to a third of all cancers harbouring a mutation(1). Normal function of this family includes regulation of transcription, cell cycle progression, growth, survival, proliferation and cell migration signalling(2). For the KRAS protein, single point mutations at positions 12, 13, and 62 have been shown to result in constitutively active protein which in turn causes over expression or increased activity in a wide range of downstream signalling pathways(3).

The high frequency of mutation in cancer has made this gene family a target of significant interest within the field of oncology, however, despite extensive efforts to exploit these proteins as potential therapeutic targets, molecules designed for RAS mutant therapies have not made progress into the clinic.

Efforts to develop new molecules to target KRAS are ongoing, with a molecule, denoted Oncrasin-1 shown in Fig. 1, being discovered in 2008 through a synthetic lethal screen with KRAS mutation in breast cancer cell lines(4). In the initial screen the compound showed selective toxicity in both a KRAS mutant cell line and a cell line with wild type KRAS which was previously resistant but became sensitive after transfection with mutant KRAS.

Further optimization of the structure has been reported, with some analogues and derivatives showing improved potency against the KRAS mutant cell lines, including analogues with fluorine at the *para*, *meta*, and *ortho* positions on the benzyl ring(5), shown in Fig. 1.

As these compounds can potentially identify mutant KRAS in different cancer types, they may have potential for development as a diagnostic imaging approach for stratification of patients to different treatment groups. This would be a valuable tool for treatment planning as the presence of KRAS mutant protein is a predictor of poor response to anti-EGFR antibody treatments such as Cetuximab and Gefetinib, due to EGFR-independent downstream signalling through this pathway(6-8).

Positron emission tomography (PET) is an imaging technique which enables non-invasive imaging of metabolic processes and biochemical function in living tissues. PET imaging currently plays a central role in the detection, staging and response assessment of cancer patients, with clinical management based on based on PET imaging results being an accepted part of cancer patient care.

Radiosynthesis of ^{18}F substituted aryl systems is currently a significant challenge in the field of radiofluorination, with limited means to effectively incorporate a radiolabel into an aryl system. Many methods which are used for synthesis of ^{19}F fluorination of aryl systems rely on electrophilic sources of fluorine, which are not easily accessible when working with ^{18}F , or protracted reaction times, which are unacceptable in the context of a 109.7-minute half-life. Preliminary evaluation of radiosynthetic methods indicated a limited number of potential precursors for fluorination reactions which could be used to label not only the *para* and *ortho* positions but also the *meta* position, which cannot be accessed through traditional electrophilic aromatic substitution radiochemistry. All three substitution patterns present a potential radiotracer target, therefore, establishing a method which could be used for radiofluorination reactions at all three positions is essential. To this end, two classes of precursors were selected for investigation.

Iodonium salt chemistry has become an area of great interest for radiofluorination of aryl systems, with many publications producing synthetic routes which have allowed access to new radiotracers through this chemistry. While these are an exciting class of precursor, not all structures are

compatible with the chemistry required to produce them, reaction conditions for labelling are very harsh, and the precursors have a limited shelf life.

In addition to the iodonium salt precursor class, a new boronic acid pinacol ester precursor has become of significant interest for aryl radio fluorination, with the functional group being known for good long-term stability and chemical resilience. The synthetic routes for these compounds are also well characterized due to their prevalence in synthetic chemistry, and the conditions for radiolabelling are not as harsh as those for iodonium salts.

Utilizing these two types of precursor, this paper presents the synthesis and automated radiolabelling of Oncrasin-like molecules for the purpose of development as PET imaging agents.

Methods

Synthesis of precursors

Coupling of indole-3-carbaldehyde and benzyl halides

A general sodium hydride deprotonation of the indole followed by addition of a benzyl halide of the appropriate substituent and substitution pattern was used for generation of cold standards as well as precursors and iodinated intermediates, as shown in Scheme 1. Yields for these compounds were typically above 70%, with the *ortho* substituted boronic acid pinacol ester derivative yielding lower at 58%.

Miyaura borylation

A standard Miyaura borylation was utilized for generation of the parasubstituted boronic acid pinacol ester precursor from an iodine substituted intermediate to afford the product in a 72% yield.

Radiochemistry

Preparation of copper catalyst

15 mg of Copper(II) triflate was dissolved in 400uL of DMF and combined with 100uL of a 50:50 mixture of DMF and pyridine. This mixture was sonicated to ensure proper solvation of the copper salt and added to the catalyst vial in the FlexLab as described in Table 2

Table 2
Flexlab set up for radiolabelling reactions

Container	Reagents
Vial 1	Eluent
Vial 2	4 mg of precursor in 500 µl of DMF
Vial 3	1 ml anhydrous acetonitrile
Vial 4	450 µl of DMF. 50 µl of Pyridine, 13 mg of Cu(OTf) ₂
Vial 5	1 ml of Acetonitrile and 1 ml of distilled water
Vial 6	
Vial 7	
Vial 8	
Vial 9	
Vial 10	
Vial 11	10 ml of distilled water
Vial 12	1 ml of ethanol
Vial 13	1 ml of saline
Vial 14	
Vial 15	
Vial 16	
Vial 17	
Vial 18	
Vial 19	
QMA seppak	Quaternary Methyl Ammonium Cartridge
Seppak A	
Seppak B	
Seppak C	
Seppak D	C18 seppak
HPLC Vial 1	
HPLC Vial 2	40 ml of distilled water
Reactor 1	1 ml anhydrous acetonitrile
Reactor 2	
Loop Vial 1	
Loop Vial 2	1 ml distilled water
HPLC eluent A	0.1% Ammonium formate
HPLC eluent B	Acetonitrile
HPLC eluent C	Water
HPLC eluent D	Ethanol

Preparation of eluent

2.3 mg of potassium triflate was dissolved in 500µL of distilled water and 500µL of acetonitrile to which 9 mg of [2.2.2] Cryptand was added. The mixture was sonicated to ensure proper solvation and mixing of components and added to the eluent vial of the Flexlab module as described in Table 2

Radiochemistry

Radiolabelling reactions were undertaken using an iPhase Flexlab automated synthesis module, shown in Fig. 3. This module is equipped with multiple reaction vessels and two HPLCs, allowing the user to undertake complex, multistep radiosynthetic reactions. Depictions of the module and interactive interface used to control it can be found in the supporting information.

4 mg of the appropriate radiotracer precursor was dissolved in 0.5 ml of DMF and combined with the previously described catalyst system with a kryptofix chelated ¹⁸F fluoride source (11.1 GBq). The reaction was heated to 140°C for 20 minutes, with purging of the reactor every 5 minutes with

atmospheric air for 3 seconds. After this time, the reaction was cooled to room temperature before dilution with 1 ml of water and transfer to a loop vial in preparation for HPLC. The reaction vessel was then washed with a further 2 ml of 50:50 H₂O:ACN to ensure full recovery of the reaction mixture before subsequent HPLC purification of the combined fractions. After initial injection of the reaction mixture, the HPLC was run for 2 minutes with a 0.1% ammonium formate in H₂O solution before switching to a gradient starting at 80:20 0.1% ammonium formate in H₂O:ACN progressing to 10:90 over 18 minutes. Products collected between 20 and 23 minutes depending on the tracer. Products were trapped using a preconditioned C18 SPE cartridge. This cartridge was conditioned with 1 ml of ethanol followed by 10 ml of water and then drying before use. For elution of the product, 1 ml of ethanol was used, with an additional 1 ml of distilled water being used to rinse the cartridge and tubing into the product vial.

Results

Synthesis of cold standards

The synthesis of cold standards was carried out for spectroscopic characterization and identification, as well as a comparison with radiolabel products for confirmation of product by radio HPLC. Synthetic procedures were undertaken as outlined in Scheme 2.

Iodonium salt precursors

The initial synthetic route to an iodonium salt precursor was envisioned as shown in Scheme 1, with a sodium hydride coupling between commercially available indole-3-carbaldehyde and the appropriately substituted iodo benzyl bromide. This intermediate would then undergo a one-pot reaction to form an iodonium salt suitable for radiolabelling. In order to determine optimal conditions, initial reactions were carried out with the *para* substituted material.

Synthesis of Compound 1 was undertaken as described in Scheme 2 and the product was obtained in quantitative yield. With Compound 1 in hand, a one-pot synthesis of the iodonium salt was trialled using reaction conditions described by Zhu, Jalalian and Olofsson(9). The resultant reaction mixture showed no indication of product formation. The characteristic proton signal correlating to the aldehyde proton at approximately 10 ppm was absent and the expected mass for the desired product

was not detected with high resolution mass spectrometry, suggesting an incompatibility between these reaction conditions and the aldehyde functional group. Literature indicated that sodium periodate(10) and peroxide(11) could also be employed as oxidants and were trialled with similar results. Other counter ions and aryl systems were also investigated, with a summary of reactants attempted with Compound 1 shown in Table 1 with none of the combinations resulting in any identifiable products other than starting material.

Table 1
Reaction conditions trialled with for iodonium salt synthesis.

Oxidant	Counter ion	Aryl System
MCPBA	OTf/OTs	<i>p</i> -Methoxybenzene
Peroxide	OTs	1,3, Dimethoxy Benzene
Oxone	OTs	1,3,5 Trimethoxy Benzene
Sodium Periodate	Acetic acid/H ₂ SO ₄	1,3,5 Trimethoxy Benzene

Attempts to carry out iodonium salt forming reactions in a two-step process shown in Scheme 4 also yielded no identifiable compounds. After determining iodonium salt conditions were not compatible with this molecule, a major product from the oxidation step of the two-step reaction was isolated via column chromatography and crystallized using vapour diffusion methods for x-ray crystallography, with the structure shown in Fig. 2.

Boronic acid pinacol ester precursors

With an iodine containing intermediate already in hand, a Miyaura borylation reaction was undertaken to provide the *para* substituted precursor in a 72% yield. As previous coupling reactions had been undertaken successfully, commercially available sources appropriately substituted boronic acid pinacol ester containing benzyl bromides were used to investigate a one-step coupling synthetic route. This method produced the desired product in yields 78%, 76% and 58% yields of the *para*, *meta*, and *ortho* precursors respectively. Product identity was confirmed through spectroscopy as well as x-ray crystal structures.

With three precursors in hand, radiolabelling experiments were undertaken.

Radiochemistry

Reaction conditions from Tredwell et al.(12) were utilized for initial radiolabelling experiments, as shown in Scheme 6. Under these conditions, no radiolabelled products were isolated.

As initial radiolabelling attempts yielded no discernible radiolabel products and previous literature

suggested that these reactions may not be suitable for automated synthesis(12), possibly due to the inert gas systems they often operate under; the mechanism of catalysis for these reactions is unclear but may operate through a Cham-Lam coupling-like oxidation cycle, which would require atmospheric oxygen that is not present in standard, inert gas flushed automated systems. The reaction was attempted again with air being purged into the reaction vessel throughout the labelling, with no improvement in radiolabel incorporation.

A 4-Methoxycarbonylphenylboronic acid, pinacol ester was utilized as a model for trouble shooting as it is chemically similar to reagents used in both the Tredwell paper and another paper authored by Mossine and coworkers(13), which had resulted in excellent yields, however under the previously stated conditions, no radiolabelling was observed.

As the catalyst system described by Treadwell was not able to produce radiolabelled products in our hands, another system, described by Mossine and shown in Scheme 7 was investigated.

This system also produced no discernible radiolabelled products at the expected retention time. Mossine and co-workers had noted poor radiochemical yields prior to their own optimization for boronic acid labelling with regards to eluents. Development of a new eluent was required for successful synthesis, which utilized a minimized quantity of potassium carbonate and potassium triflate in combination with [2.2.2] Cryptand.

Attempts to carry out the radiosynthesis with the model system utilizing other standard eluents such as bicarbonate and tertbutyl amine were unsuccessful.

To determine if the eluent was the limiting factor in the radiosynthesis, a synthesis was carried out without fluoride isolation, with evaporation of the ^{18}O water being performed prior to the labelling reaction, which yielded minor new radiolabelling products.

Adoption of a potassium triflate eluent system afforded radiolabelling of the model system as the major radiolabel product. Further optimization of the eluent showed that the preconditioned QMA cartridge used for an ^{18}F -FDG synthesis contained enough bicarbonate for labelling and so this was removed from the eluent. When using QMA cartridges which had been reconditioned after initial use,

significant variability was observed, so this was avoided for future synthesis.

Having successfully produced a radiolabelled molecule in the model system, the BpinKAM001 system was revisited, utilizing the revised catalyst system and new eluent, with successful product formation being achieved. HPLC purification of the radio peak from the reaction mixture was undertaken in the Flexlab module and confirmed to be the desired radiotracer by registration with the cold standard peak retention time, as shown in Fig. 4. Using these conditions radiolabelling of the remaining BpinKAM002 and BpinKAM003 compounds was undertaken successfully, with HPLC traces shown in Fig. 5 and Fig. 6

Decay corrected yields for the purified tracers were 10–12% (n = 5), 10–25% (n = 3) and 12–18% (n = 3) for ^{18}F KAM001, ^{18}F KAM002 and ^{18}F KAM003 respectively.

Discussion

Synthesis of iodonium salt precursors

Reactions to attempt to form an iodonium salt precursor were incompatible with the indole scaffold contained in the Oncrasin molecule as the indole structure is preferentially being oxidized rather than the iodine in the benzyl ring. This process likely occurs with all of the oxidants utilized in the reactions undertaken, with a wide range of intermediates being produced, resulting in complex mixtures of products. The structure obtained from the peracetic acid reaction suggests a multi-step Baeyer-Villiger oxidation, resulting in a ring opening reaction which is consistent with oxidation processes proposed by Marchand et. Al.(14).

This understanding of indole incompatibility with the oxidation required for iodonium salt forming reactions is a significant development for ^{18}F radiochemistry, as indoles are an important biological scaffold and are present in many structures which may be investigated as PET imaging agents including amino acids, peptides and small molecule drugs.

As the indole structure is not amenable to the required oxidation conditions for a direct iodonium salt synthesis, attention was directed towards the boronic acid pinacol ester precursors.

Pinacol boronic esters precursors were readily produced through two synthetic routes, with good yields for both routes, proving to be a superior precursor class for this indole containing compound.

The lower yield of the ortho product can be explained by a steric clash observed in the crystal structure of this product, which would make nucleophilic attack of the halogen less favoured, hindering product formation.

Radiochemistry

Yields for these Oncrasin-like molecules are somewhat low compared to other literature reports, the radiochemical yield was in excess of what is required for preliminary biological evaluation. Both Mossine and Treadwell papers noted that precursors containing nitrogen produced lower radiochemical yields when utilizing the copper catalysed labelling. This lower yield could potentially be due to the precursor competing with pyridine in forming the catalyst system, both deactivating the catalyst and sequestering precursor so that it is not available for radio fluorination. All tracers were shown to have a radiochemical purity greater than 99%, with a representative run of the ^{18}F KAM002 producing a specific activity of 1.05 GBq/ μmol .

Conclusions

Synthesis of Oncrasin-like molecules was attempted through both an iodonium salt precursor and a boronic acid pinacol ester precursor. Reaction conditions for direct synthesis of an iodonium salt precursor were not compatible with the indole scaffold in the structure. Boronic acid pinacol ester precursors were successfully synthesized and radiolabelled utilizing modified reaction conditions reported by Mossine and co-workers. Identifying the incompatibility of the indole scaffold and common iodonium salt-forming reaction conditions is an important contribution to field of radiotracer development. Synthesis of these radiotracers will allow for further work to determine suitability of Oncrasin-based molecules as potential diagnostics for cancer detection and tumour type differentiation.

Abbreviations

RCY

Radio chemical yield

RAS

Rat Sarcoma

KRAS

Kirsten Rat Sarcoma

EGFR

Epidermal Growth Factor Receptor

PET

Positron Emission Tomography

DMF

Dimethylformamide

ACN

Acetonitrile

HPLC

High Performance Liquid Chromatography

GBq

Giga-becquerel

SPE

Solid Phase Extraction

MCPBA

Meta-chloro perbenzoic acid

OTf

Triflate anion

OTs

Tosylate anion

QMA

Quaternary Methyl Ammonium

FDG

Fluorodeoxy glucose

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Crystal structure generated and/or analysed during this work are available in the Cambridge Crystal Structure Database repository. <https://www.ccdc.cam.ac.uk/>

All other data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

AM undertook all synthetic procedures for compounds with advice from JW and drafted the publication. YG and UA assisted with radiochemistry development. AS provided advice on clinical relevance for tracer development. UA, JW and AS reviewed the paper for publication.

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Figures

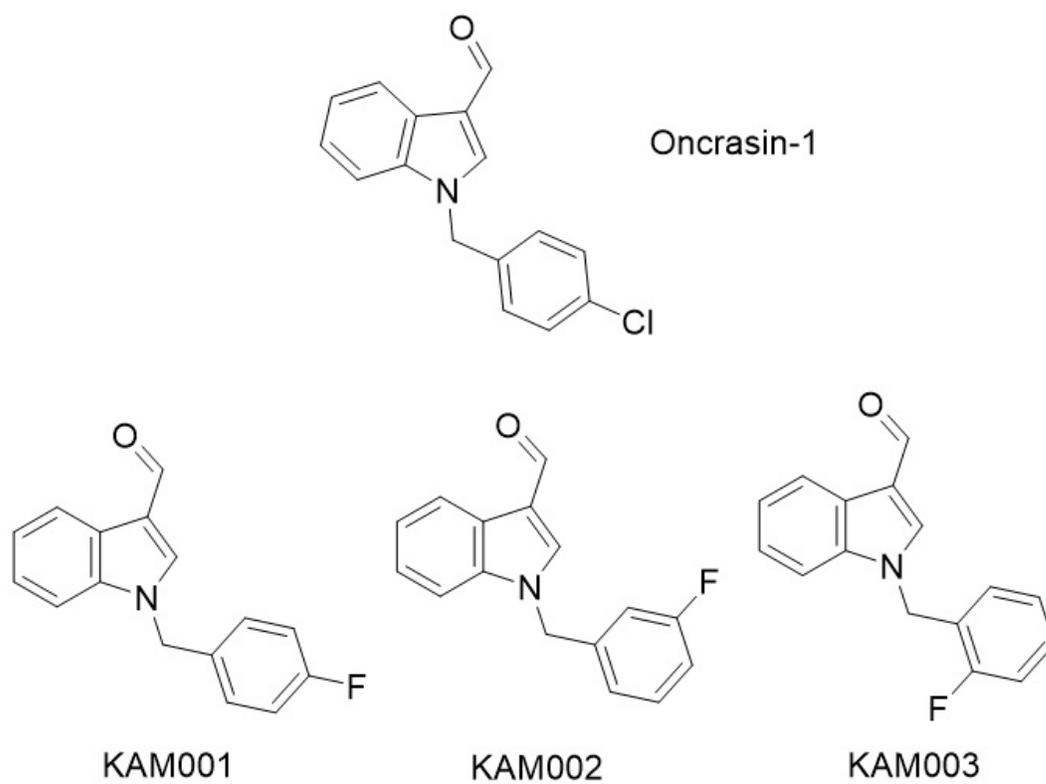


Figure 1

Parental compound, Oncrasin-1, and 3 fluorinated derivatives synthesized.

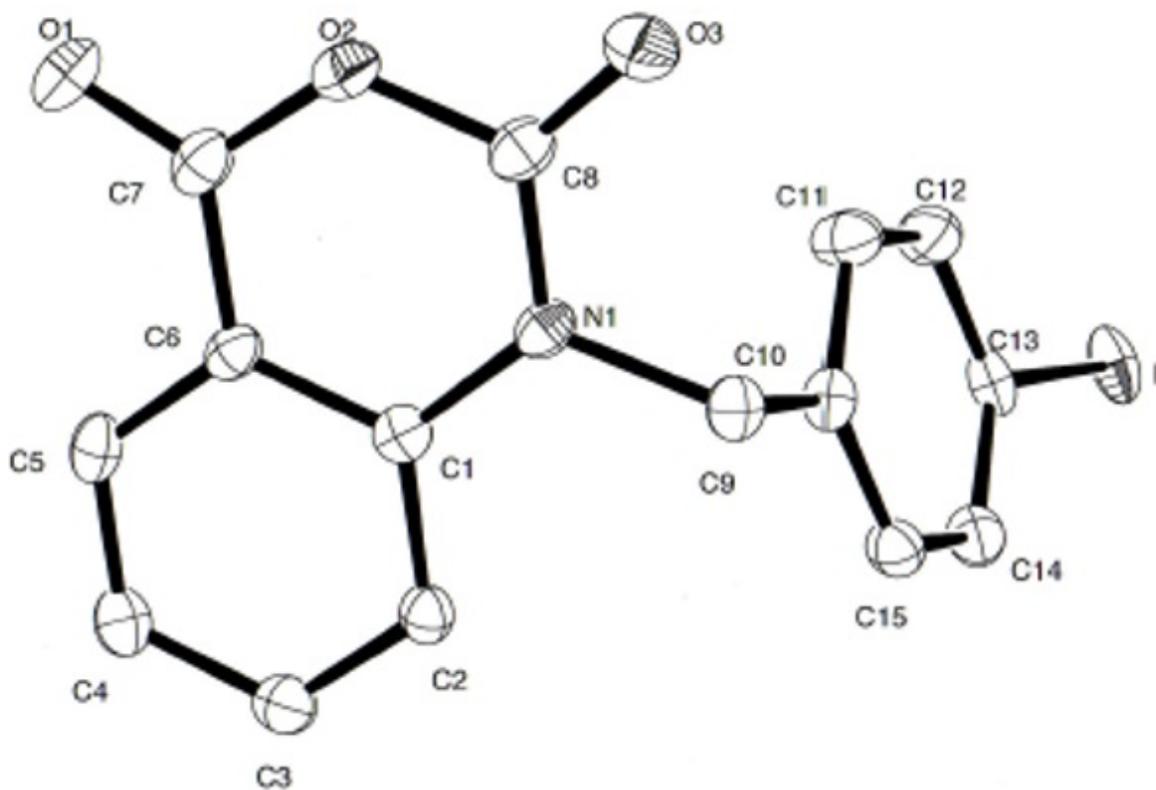


Figure 2

Figure 2: Crystal structure of a major product Step 1 in Scheme 4.

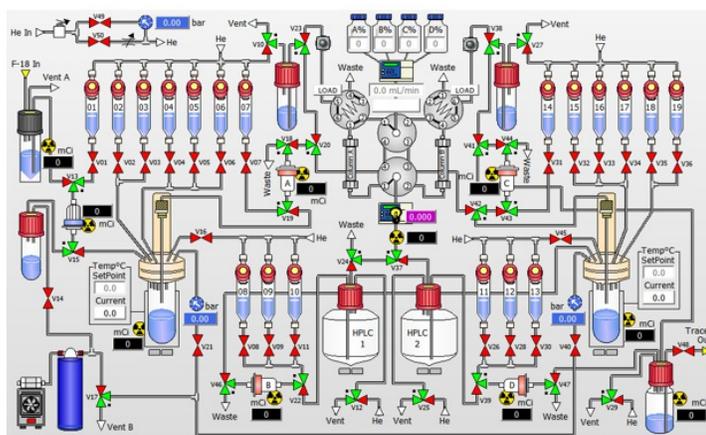


Figure 3

Flexlab module (left) and interactive control interface (right)

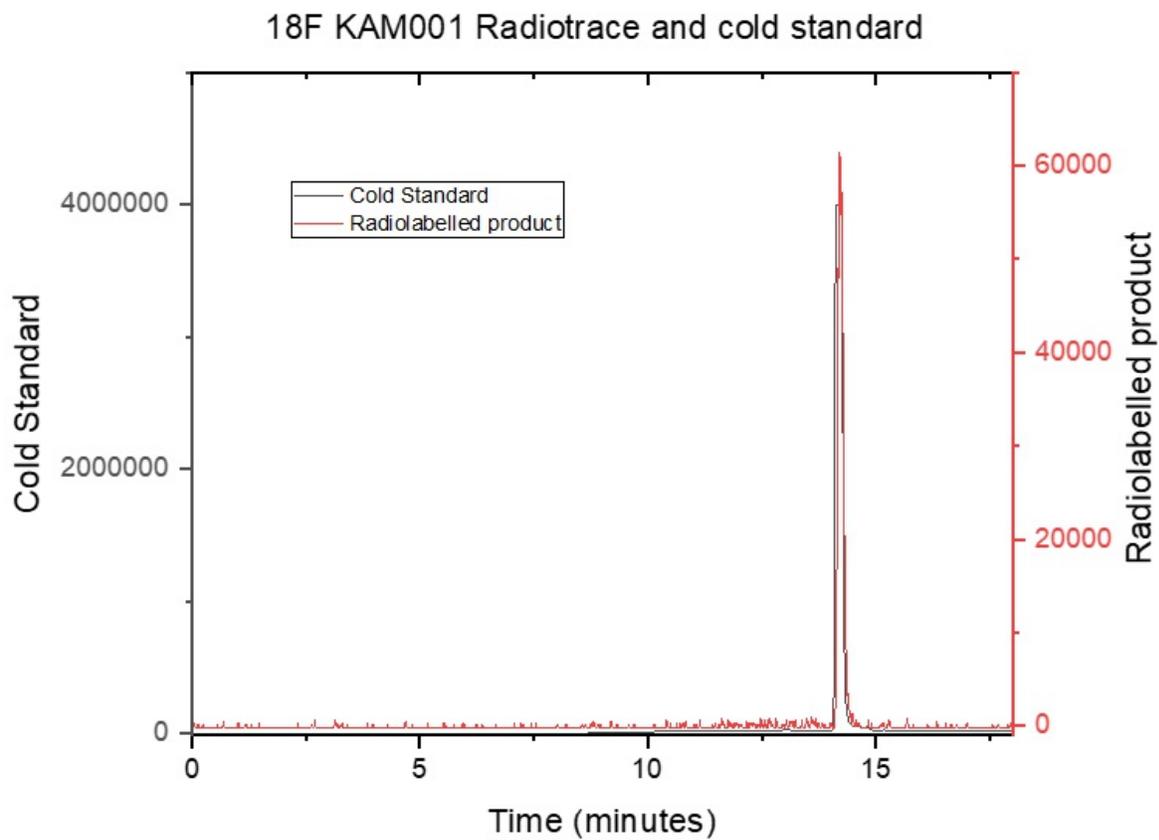


Figure 4

18F KAM001 identity confirmation using cold standard retention.

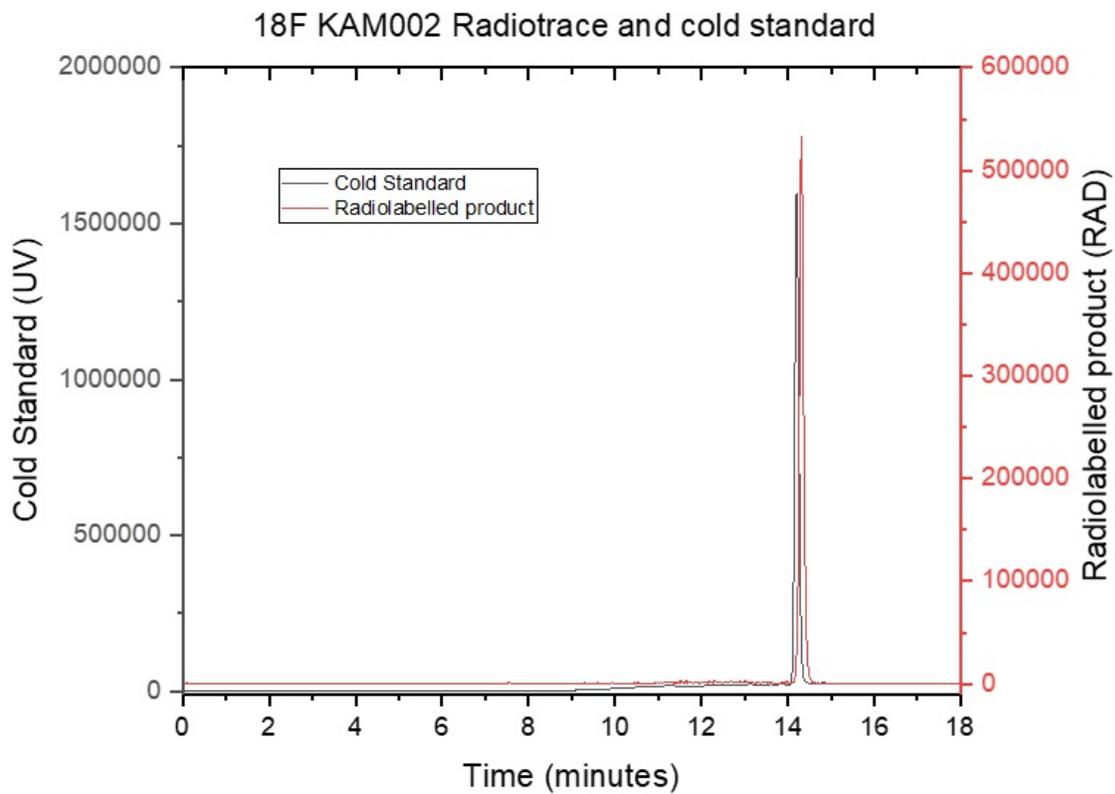


Figure 5

18F KAM002 identity confirmation using cold standard retention.

18F KAM003 Radiotrace and cold standard

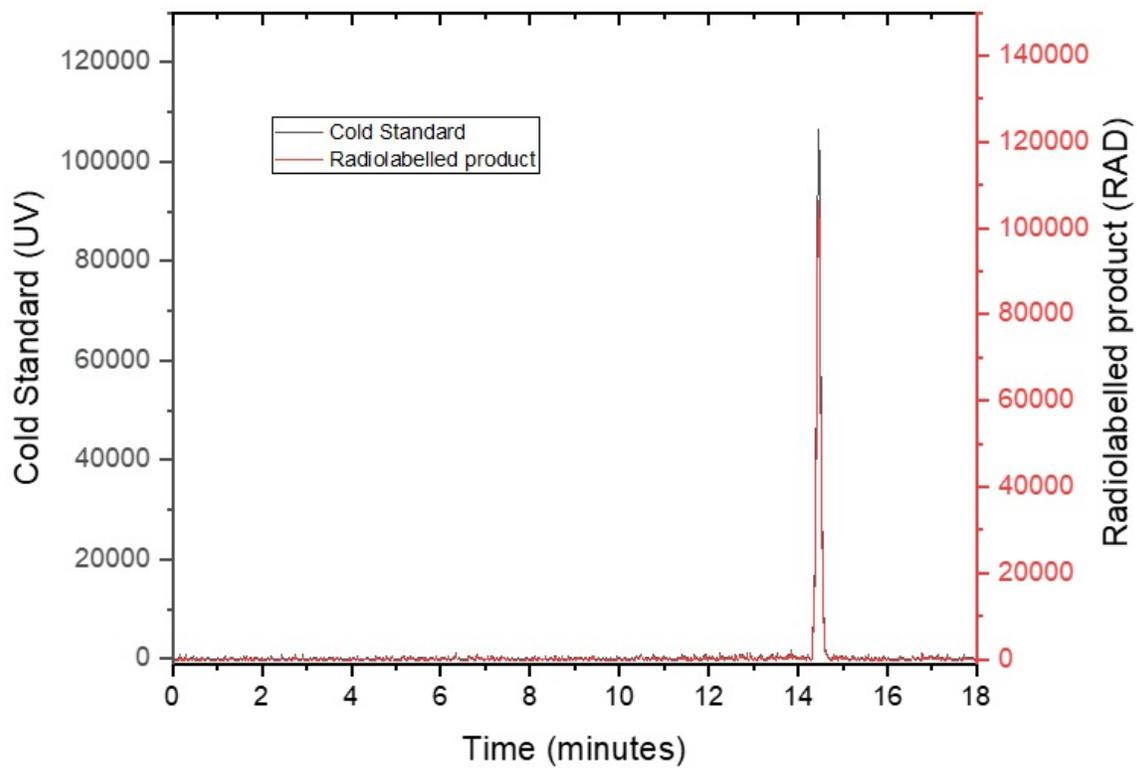
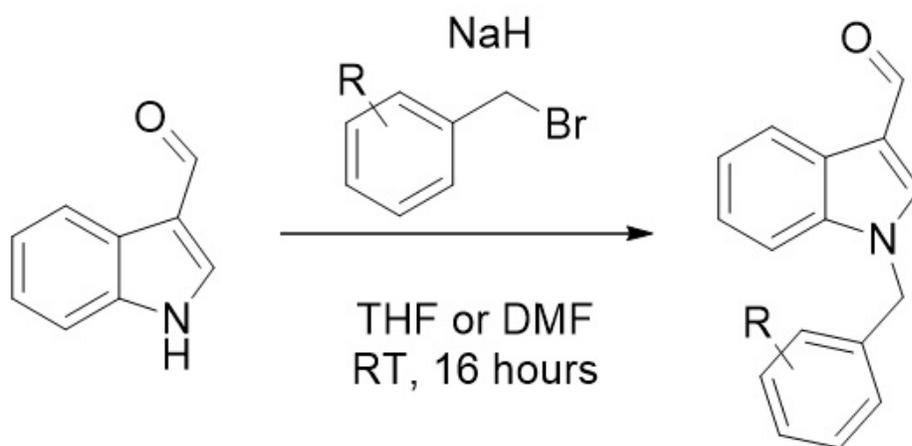


Figure 6

18F KAM003 identity confirmation using cold standard retention.



R = F, I, or Bpin substitution at *o*, *m*, or *p* position

Figure 7

Scheme 1: Standard coupling conditions for indole-3-carbaldehyde and various benzyl bromides.

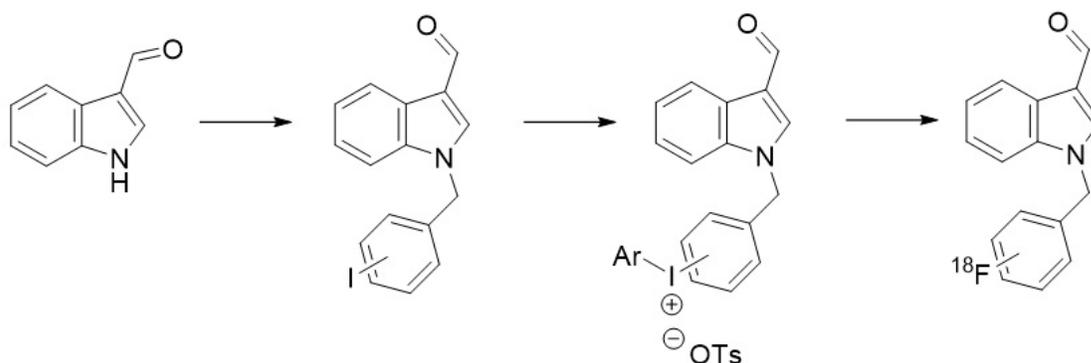


Figure 8

Scheme 2: Proposed synthetic route for iodonium salt synthesis and subsequent labelling

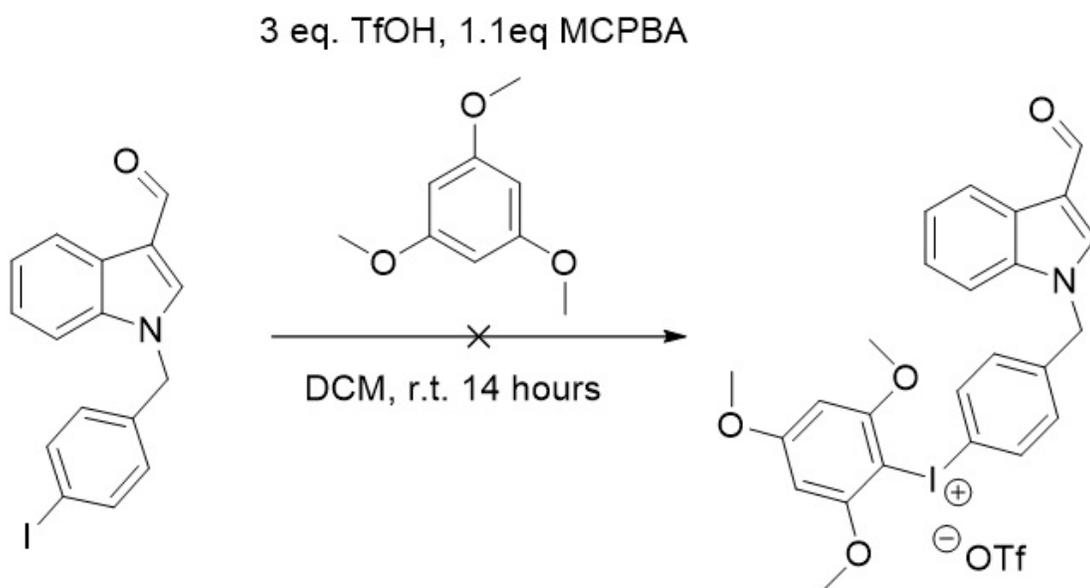
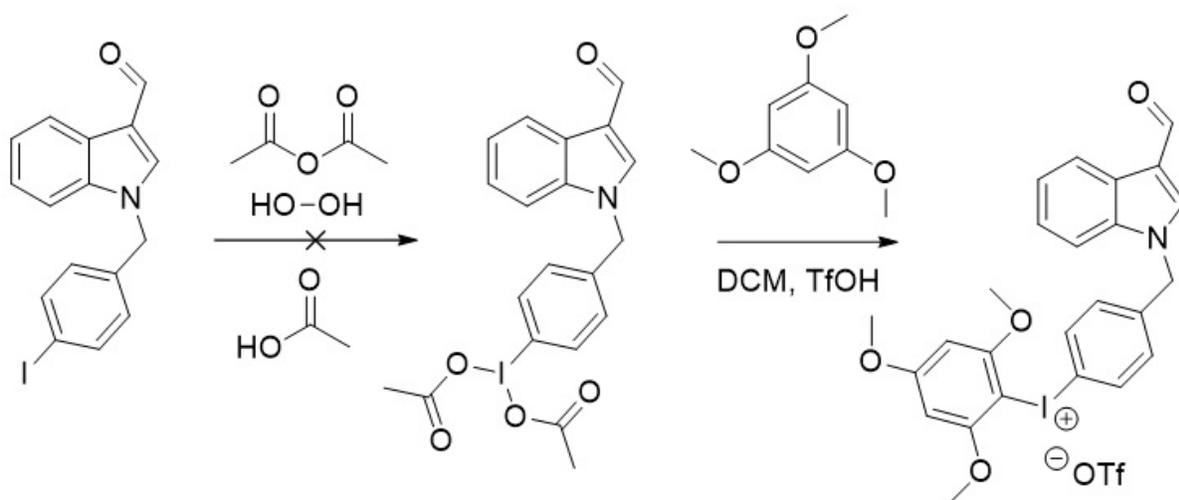


Figure 9

Scheme 3: Attempted initial iodonium salt synthesis



1) Formation of a
'diacetoxy-iodo' intermediate

2) Conversion to the desired
aryl-iodonium salt system

Figure 10

Scheme 4: A 2-step approach to iodonium salt synthesis.

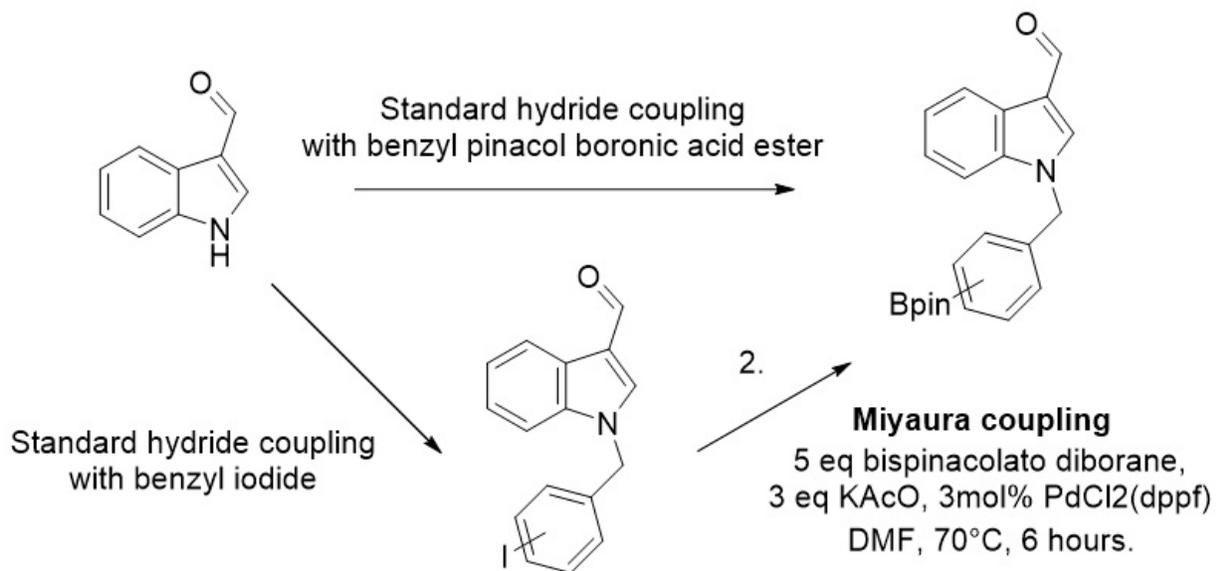


Figure 11

Scheme 5: Synthesis of boronic ester precursors via two routes

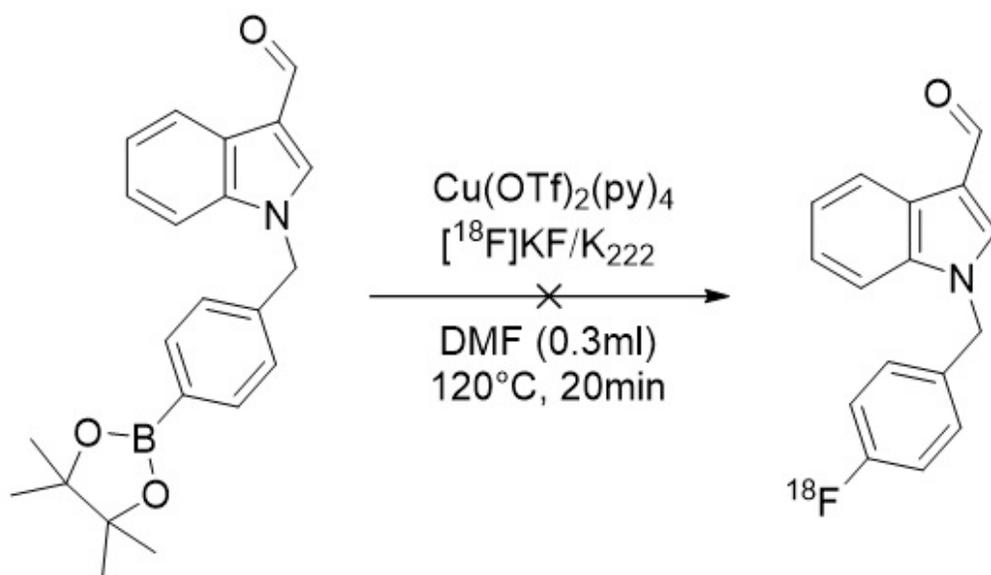


Figure 12

Scheme 6: Initial radiolabelling trial conditions utilizing Treadwell conditions.

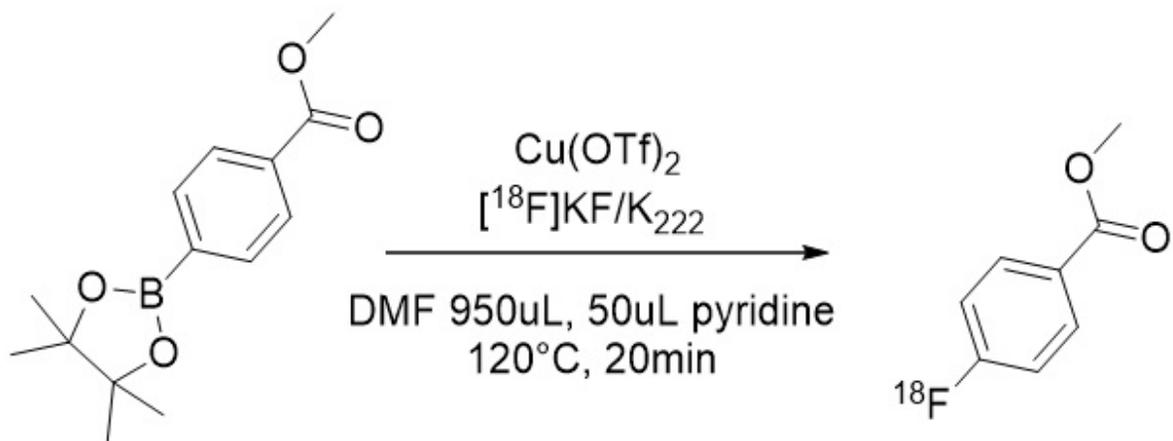


Figure 13

Scheme 7: Radiolabelling conditions used for labelling of model system

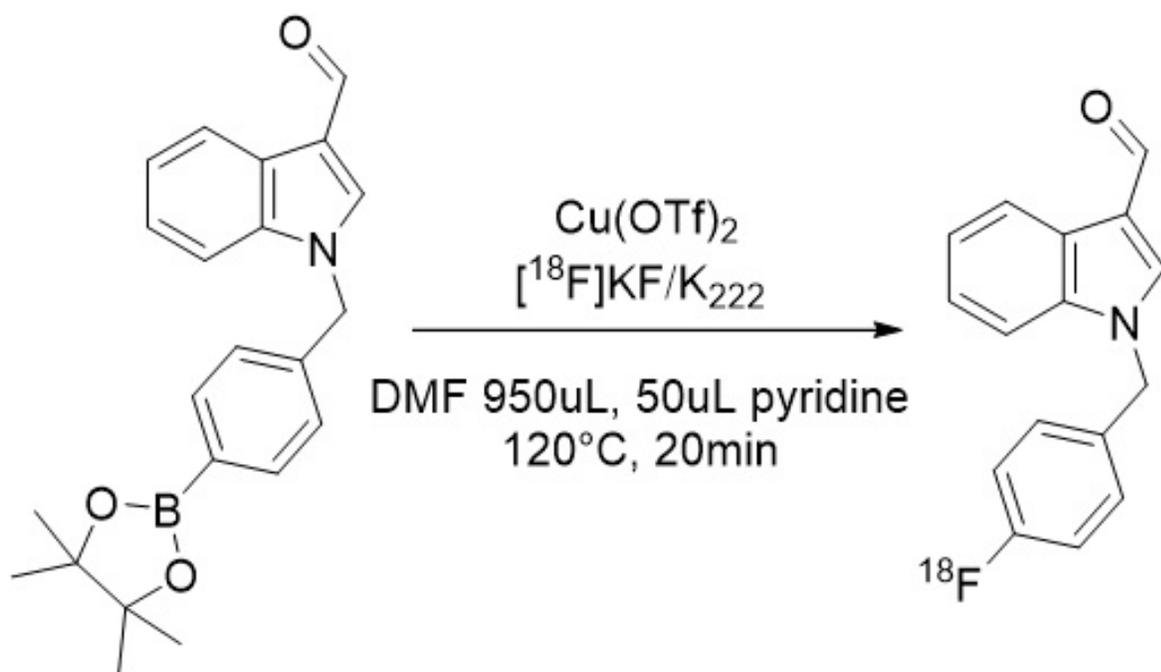


Figure 14

Scheme 8: Radiolabelling conditions for KAM001

Supplementary Files

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