nature portfolio

Peer Review File

Late-Stage (Radio)Fluorination of Alkyl Phosphonates *via* Electrophilic Activation

Corresponding Author: Dr Zijing Li

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Zhang, Feng, Mou et al. describes a novel late-stage fluorination method for alkyl phosphonates via electrophilic activation. The authors demonstrate its utility for 18F-radiolabeling of several biomedically relevant compounds. Essentially, pyridine salts are also a leaving group, and this work is still an expansion of the leaving group from the previous work(https://doi.org/10.1038/s41467-019-08953-0). The previous work was the final step in synthesizing 18F compounds, and this work is also the final step in synthesizing 18F compounds, only with different precursors. The synthesis of precursors is not a rate limiting step for radiolabeling. Therefore, compared to the previous work of the research group, this work lacks innovation. And there are several issues that need to be addressed:

1. More details are needed on the radiochemistry. The authors state radiochemical conversions of 43-77% were achieved, but do not provide radiochemical yields or specific activities for the 18F-labeled compounds. This information is critical to assess the suitability of the method for radiopharmaceutical production.
2. Stability is the focus of research on P-F exchange method. If the stability of the tracer is poor, it cannot be applied to the design of tracers. This should be the focus of innovative research on P-F exchange method. It is evident from Figure S20 that the tracer is defluorinated. Therefore, this study should focus on the applicability of this labeling method. This study only investigated the in vitro stability of one tracer, which is not sufficient to demonstrate the stability of the tracer. Therefore, information on in vitro and in vivo stability, including defluorination, should be included.
3. The mechanistic studies, while informative, are not fully conclusive. Isolation and more complete characterization of the proposed phosphonium intermediates would provide stronger support for the proposed mechanism. Computational studies alone are not sufficient.
4. The manuscript would benefit from more context on how this method compares to and improves upon existing late-stage 18F-fluorination approaches for alkyl phosphonates and other substrates. A more thorough discussion of advantages, limitations and scope is recommended.
5. Some additional controls should be included, such as 18F-labeling of non-alkyl phosphonate precursors, to confirm selectivity and functional group tolerance.
6. It's unclear if this radiofluorination approach works for more complex biologically relevant molecules beyond the few examples shown.
7. The manuscript would benefit from more quantitative discussion of how this method improves upon existing approaches in terms of radiochemical efficiency, synthesis time, automation capability etc.

Reviewer #2

(Remarks to the Author)
This is a highly interesting work that develops a novel method for synthesizing organic fluorophosphines. Through electrophilic activation by Tf2O and optimized conditions, the reaction completes within 15 minutes and achieves a yield of 99%, making it more efficient and convenient than traditional strategies. Furthermore, the team applied this new methodology to the late-stage radiofluorination of a broad range of dialkyl and monoalkyl phosphonates, and they tested it in vivo. Overall, the presented work is of significant interest, well-executed, and I recommend accepting this paper for publication. However, there are a few concerns and questions that, if addressed, would further solidify the authors' work:
1. The limitations of prior work presented in Fig. 1a are confusing and should be clarified. What do the blue and red circles represent, and why are they considered limitations of the previous work?

2. When designing and synthesizing alkyl phosphonate precursors for 18F-tracers, it is crucial to preserve the core activity or function of the original molecules. The compounds [18F]31a-35a lack characterization after the addition of the organic fluorophosphine moiety. To strengthen the radiofluorination strategy, please provide the cLog P and other drug property parameters of the original molecules and the 18F-tracers. Additionally, it would be beneficial to include the binding affinity of both the original molecules and the 18F-tracers to their target proteins.
3. The authors claim that P-benzyl fluorophosphonamide exhibits excellent stability under both in vitro and in vivo conditions. Please provide the stability data for both in vitro and in vivo conditions to support this claim.

Reviewer #3

(Remarks to the Author)
The manuscript entitled “Late-Stage (Radio)Fluorination of Alkyl Phosphonates via Electrophilic Activation”, authored by Zhang et al., describes a method to make alkyl fluorophophonates from activated phosphinates in situ, extending its application to 18F-radiochemistry. This non-radioactive approach as well as radioactive version to fluorinate phosphorus containing moiety in organic entities would be useful for many purposes, as significant level of investigation has been made. Although the reported procedure may find useful application, the substrate scope to reflect electronic nature and steric, apart from complexity and numbers of substrate employed, in this study seems insufficient for applications in organic community in general. As an example, instead of adopting similar electronic nature of substituent as described in Fig 4. (a) (para-pattern, halogens, EWG), the inclusion of one or two meta-EWG, EDG and ortho-EWG, EDG would be beneficial to readers interested in this methodology. Therefore, it is strongly suggested to implement additional substrates (with diverse substitution patterns and electronics) to prove the generality of proposed method, ideally with one or two examples of highly functionalized substrates. Refer to other points described below for improvement. Some aspects of the work should be explained in greater detail in both main text and supporting materials. After those have been fully addressed, I would expect this article to meet the stringent publication criteria of Nature Communications. I would suggest improving the manuscript before acceptance.

Points to improve manuscript:
1] Since electrophilic activation appears to be a crucial step in this transformation and the rationale behind this activation is referenced, it is unclear why Tf2O is used to activate the dialkyl phosphonate. Is the 1.5 equivalent of Tf2O necessary for a successful reaction? Unlike the non-radioactive fluorophosphonate synthesis, it is important to avoid using excess activator in the production of 18F-radiotracers to simplify the downstream quality control workload (such as chemical and radiochemical purity).

2] The reason for using an N-heteroaromatic base was not fully explained. Have common bases like Et3N or DIPEA been considered for this purpose? What reasons exist for not including these bases in this transformation?

3] In this transformation, the fluoride source was identified as Et3N3HF and used throughout the experiments. In 18F-labeling, the 18F-K222/K2CO3 complex was utilized as the 18F-fluorine source. Since the nonradioactive modeling reaction was performed on a small scale (i.e., 0.2 mmol) and characterized with NMR, it would be beneficial to demonstrate the KF/cryptand combination (a similar nucleophilic fluoride source as used in radiochemistry) to improve nucleophilicity and solubility in organic solvents.

4] In certain cases, 31P NMR was utilized to determine the chemical yield of a reaction. It is recommended to include specific experimental details for accurate yield calculation with 31P qNMR. It is common practice to assess chemical yield using heteronucleus like 19F. Apart from the natural abundance of each nucleus, differences in relaxation time and delay parameters between 19F and 31P NMR could potentially lead to varying quantitative NMR results.

5] In proposing a mechanism with computational observation (Figure 3), the oxidation state at the phosphorus center might influence the overall chemical intermediacy and reaction pathway. Can you propose a relationship between the oxidation state of central phosphorus and the outcomes of electrophilic activation leading to final product (such as TS-1, INT-1, INT-2) in terms of stability, geometry, and reactivity? This could be very helpful to support the proposed mechanism rather than just showing the reaction progressing path.

6] In Figure 1, (a), readers may mistakenly think that 18F-labeled alkyl phosphates come from 18F-DAST, even though it is mentioned in parentheses, which is an electrophilic radiofluorination. This requires clarification.

7] Throughout the manuscript, the compound numbering system needs to be more organized. Preferably with brackets and other symbols or alphabets. Such as 1b, rac-1a, INT-1’ (should be in Figure 3(b)), and many more are not properly described in the main text and have caused confusion. Coordinate compound numbering with the supporting information properly as well.

8] 31P NMR in Figure 3 (b) is very hard to read. It would be nice to redraw the figure in order to be read easily (especially chemical shift in ppm).

9] Authors are advised to adhere to the guidelines related to radiochemical notation. The terms "RCC" and "RCY" are confusing. Refer to the guideline and modify the expression accordingly (see, Nucl Med Biol 2017, 55, v; ibid.). 2019, 71, 19; ibid. 2021, 93, 19).

10] In Supplementary Materials S17: There was no solvent used to make 1. Is this originally solvent-free reaction? Please specify.

11] In Supplementary Materials S10: It would be helpful to include the mixed base stoichiometry for experimental details. Additionally, in S14, the ratio between alcohol and thiol should be clearly specified.

12] In Supplementary Materials S9: Is there a specific reason to track the reaction progress using 31P NMR? Can we monitor the reaction progress effectively using traditional TLC instead?

13] In Supplementary Materials S15, what is the role of DMSO? The scheme in S15 is misleading because it appears to depict DMSO as a solvent.

14] In the Supplementary Materials S46, it is generally known that Cs2CO3 has very limited solubility in neat MeCN (a relatively large amount of Cs2CO3 is used for 100 µL of MeCN). Does this impact the overall RCC as indicated in Table S3, entry 2?

15] It is recommended to include the HPLC analysis of the crude reaction mixture, including UV and gamma traces, at least for several reactions. Due to the complexity of the two-stage one-pot reaction, the chromatographic profile of the radiofluorination mixture is expected to be intricate. This aspect is crucial in real-world PET radiotracer production because it directly affects the chemical purity of radiopharmaceuticals meant for human use. The manuscript does not clearly specify this, and it is challenging to discern from the provided UV trace, which only includes the co-injection of a reference standard in the HPLC chromatogram. The cleanliness of this reaction is crucial for determining the purification effort needed in the use of the short half-life (approximately 110 minutes) radioisotope.

16] In Supplementary Materials S54 and S55, based on the HPLC and radio-HPLC chromatograms of [18F]35a provided, a significant amount of radioactive byproduct is consistently formed (confirmed by three runs and UV trace) near the product peak. Is it possible that sulfide is influencing the production of this undesired radioactive byproduct? Alternatively, can you adjust the HPLC conditions to separate the overlapping sections? This issue is also observed with [18F]36a and [18F]AquaF-Flurpridaz.

17] In Supplementary Materials S62, please provide specific details regarding the assay concentration, not just a range. If the protocol was not developed in the laboratory, include a reference to enable replication of the experiment.

18] In Supplementary Materials S62, it is also advisable to conduct a stability test of the compound using UV trace (refer to Figure 19S, A and B). This is important because under radio-HPLC conditions, only radioactive (gamma) signals can be detected. Therefore, if there is degradation of the non-radioactive chemical entity (as indicated by the UV trace), it does not necessarily imply that [18F]BFPA-E[c(RGDyK)]2 is stable for further biological assessment.

19] In Supplementary Materials S59, 60: As previously discussed, employing a two-stage one-pot method on a large scale would be advantageous for the radiopharmaceutical community. To enhance understanding of the radiofluorination process, it is suggested to include a preparative HPLC chromatogram of crude reaction mixture before purification and an analytical HPLC chromatogram to evaluate the integrity of the reaction. This approach is more beneficial than just relying on a correlation curve to calculate molar activity.

20] Throughout the main text and supplementary material, the separate use of 19F- and 18F- notation is unnecessary. Non-radioactive reactions can be simply described as fluorination, fluoro-, etc., while only radioactive reactions or products should be noted with 18F-.

Reviewer #4

(Remarks to the Author)
Comments:
In this manuscript, Li and coworkers report the room-temperature-driven nucleophilic fluorination to construct fluorophosphines via active phosphine intermediates. This method enables the late-stage (radio)fluorination of broad dialkyl and monoalkyl phosphonates. The mechanism of this late-stage fluorination was fully explored by experimental and computational studies. The substrate scope is wide, and the fluorination of many bioactive molecules can also be obtained. Furthermore, radiofluorination of medically significant 18F-tracers and synthons are completed, and corresponding PET experiments were conducted. Because of the importance of the late-stage (Radio)fluorination of alkyl phosphonates, I believe that the current investigation is truly useful and of board interests of the chemical society. Nevertheless, several issues are required to be addressed before acceptance.

Point 1: In the introduction, the authors should give more reasons why chosen N-heteroaromatic bases (line 82-83).

Point 2: In Figure 2a, which N atom will coordinate to P(V) in a5 and a6? I think the lone pair should be drawn on the other N atom.

Point 3: In Figure 2c, Why iPrO group has a higher yield than EtO group? In Figure 3c, OTf- attacking on the methylene of the ester is the rate-determining step. iPr group has a larger steric hindrance than Et group. I speculate that the TS-3 of iPrO

group should be higher than that of EtO group.

Point 4: Could you please help me explain the content in lines 193 to 196? I don't fully understand now.

Point 5: Since the scan of the P-N distance indicates that TS-2 may not exist in Figure S30, TS-2 is unnecessary in Figure 3c.

Point 6: For 26a, in my opinion, the Ph group in N-methylaniline is an electron-withdrawing group. Therefore, the N-methylaniline group should increase the electrophilicity of P-center rather than decrease it? Perhaps a new explanation is needed for why 26a fails to generate the desired product.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The author responded well to my question. I have no further questions.

Reviewer #2

(Remarks to the Author)
The authors have addressed concerns, and the referee suggests accepting the manuscript without further revision.

Reviewer #3

(Remarks to the Author)
The revised manuscript by Zhang et al. properly addresses issues associated with the reviewers' comments and suggestions. The methodology proposed in this manuscript will enable the efficient generation of diverse alkyl phosphonates through the electrophilic activation of precursor, as well as its relevance to radiochemical applications. I think the manuscript has significantly improved and is worthy of publication in Nature Communications, particularly for its broader audience, including those in the organic and radiopharmaceutical fields.

Reviewer #4

(Remarks to the Author)
The author has thoroughly addressed the reviewers' comments, and I recommend that this manuscript be accepted for publication in Nature Communications. There is only one minor issue: in response to the final comment, it should be clarified that it is the -Ph group in N-methylaniline that is electron-withdrawing, rather than N-methylaniline itself being electron-withdrawing.

Reviewer #1

The manuscript by Zhang, Feng, Mou et al. describes a novel late-stage fluorination method for alkyl phosphonates via electrophilic activation. The authors demonstrate its utility for ^{18}F-radiolabeling of several biomedically relevant compounds. Essentially, pyridine salts are also a leaving group, and this work is still an expansion of the leaving group from the previous work (https://doi.org/10.1038/s41467-019-08953-0). The previous work was the final step in synthesizing ^{18}F compounds, and this work is also the final step in synthesizing ^{18}F compounds, only with different precursors. The synthesis of precursors is not a rate limiting step for radiolabeling. Therefore, compared to the previous work of the research group, this work lacks innovation. And there are several issues that need to be addressed:

Re: Thank you for the comment. We address the innovation question as outlined, which is also succinctly articulated in the revised introduction section.

This work introduces a novel nucleophilic fluorination method *via* electrophilic activation to construct widely employed fluorophosphines, demonstrating high selectivity for alkyl phosphonate substrates, particularly the prevalent phosphonate ethyl esters. Compared to previously reported ^{19}F-fluorination methods, this approach enables the direct production of fluorophosphonates from phosphonate ethyl esters, bypassing the need for leaving group-containing reactants through multi-step and harsh synthesis. This results in a more straightforward, milder, and cost-effective route to biomedical inhibitors. In contrast to the earlier ^{18}F-labeling method utilizing $^{18}F/^{19}F$-isotope exchange (*Nat Commun* **10**, 989, (2019), https://doi.org/10.1038/s41467-019-08953-0), our new method in ^{18}F-labeling application achieves higher molar activity utilizing readily available and separable phosphonate ethyl ester precursors than the isotope-exchange method that involves inseparable precursors.

1. ***Simplified and efficient synthesis of fluorophosphonate inhibitors for biomedical use***. Traditional synthesis of fluorophosphines, such as FPBP, requires laborious multi-step processes with intermediate purification. This work offers a direct, atom-economic synthesis of fluorophosphines, achieving a notably higher yield of up to 78%, simplifying the preparation of bioactive fatty acid amide hydrolase inhibitors.

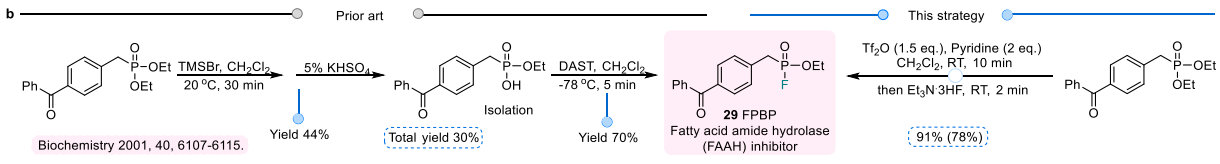


Figure 5b, An exemplification of the method's application in the synthesis of FPBP, a fatty

acid amide hydrolase inhibitor.

2. ***Versatility of the substrates.***
   The method features high selectivity in alkyl phosphonates fluorination, circumventing the stringent conditions (such as strong acid/base reflux and hazardous reagents) that typically restrict substrate diversity. This innovation allows for the fluorination of a wide array of alkyl phosphonates, including dialkyl, monoalkyl, mixed phosphonates, and amino-substituted alkyl phosphonamides, thus expanding the synthetic horizon for fluorophosphines research.
3. ***Improved molar activity for ^{18}F-labeling.*** This work describes a nucleophilic substitution method for radiolabeling with [^{18}F]F^{-}, achieving a feasible separation of precursor and ^{18}F-labeled product. This approach yields a significantly higher molar activity (251 ± 12 GBq/μmol) compared to our previous ^{18}F/^{19}F-isotope exchange (2.22–4.81 GBq/μmol), which is highly critical for receptor-binding small-molecule imaging tracers (the new "**Late-stage ^{18}F-fluorination of ^{18}F-tracers**" section).
4. ***Enlarged precursor scope and improved pharmacokinetic property for ^{18}F-tracers***. The expanded precursor scope enables direct radiolabeling applications. PET imaging studies has shown that the “BFPA” building block exhibit faster background clearance (30 min) compared to DBPOF (*Nat Commun* **10**, 989, (2019), https://doi.org/10.1038/s41467-019-08953-0) (the new "**Late-stage ^{18}F-fluorination of ^{18}F-synthons**" section).

1) More details are needed on the radiochemistry. The authors state radiochemical conversions of 43-77% were achieved, but do not provide radiochemical yields or specific activities for the ^{18}F-labeled compounds. This information is critical to assess the suitability of the method for radiopharmaceutical production.

Re: Thank you for your constructive comment. In the revised manuscript, **Figure 6a** now includes the molar activity (A_ms) for compounds [^{18}F]**37**, [^{18}F]**40**, [^{18}F]**42**, [^{18}F]**43**, and [^{18}F]**45**, with the detailed A_m information presented in new **Figure S31** and **S33** of the revised Supplementary Information. We have included the corresponding radioTLC traces in the new **Figure A1**–**A6** (in the **10. Appendix** section of the revised Supplementary Information) to illustrate all the radiochemical conversions (RCCs). We have also provided the original HPLC traces for all ^{18}F-labeled compounds to detail the separation process in the new **Figure S2**–**S27**.

*2) Stability is the focus of research on P-F exchange method. If the stability of the tracer is poor, it cannot be applied to the design of tracers. This should be the focus of innovative research on P-F exchange method. It is evident from **Figure S20** that the tracer is defluorinated. Therefore, this study should focus on the applicability of this labeling method. This study only investigated the in vitro stability of one tracer, which is not sufficient to demonstrate the stability of the tracer. Therefore, information on in vitro and in vivo stability, including defluorination, should be included.*

Re: Thank you for your constructive comment.

We conducted *in vivo* stability investigations with [^{18}F]BFPA-E[c(RGDyK)]$_2$ (re-synthesized and purified to achieve an RCP > 99%) by analyzing blood and urine samples, as well as conducting PET imaging. As illustrated in the new **Figure S35c** and **Figure S35d**, the tracer maintains over 94% stability in blood and over 92% stability in urine at 60 min post intravenous injection. Compared to the previously reported NHS-modified ^{18}F-FPRGD2 (*Eur J Nucl Med Mol Imaging* **34**, 1823–1831, (2007), https://doi.org/10.1007/s00259-007-0427-0, **Cited Figure**), [^{18}F]BFPA-E[RGDyk]$_2$ shows superior *in vivo* stability in blood (over 94% stability) in contrast to 74.2% for ^{18}F-FPRGD2 at 60 min post-intravenous administration. The updated microPET images are now shown in the new **Figure S38**, and no defluorination can be observed in **Figure S38** while only acceptable minor bone uptake can be detected under block conditions. Above all investigations strongly approve that [^{18}F]BFPA-E[c(RGDyK)]$_2$ exhibits good *in vivo* stability. The *in vitro* stability test has been repeated, too, as shown in the new **Figure S35a** and **Figure S35b**.

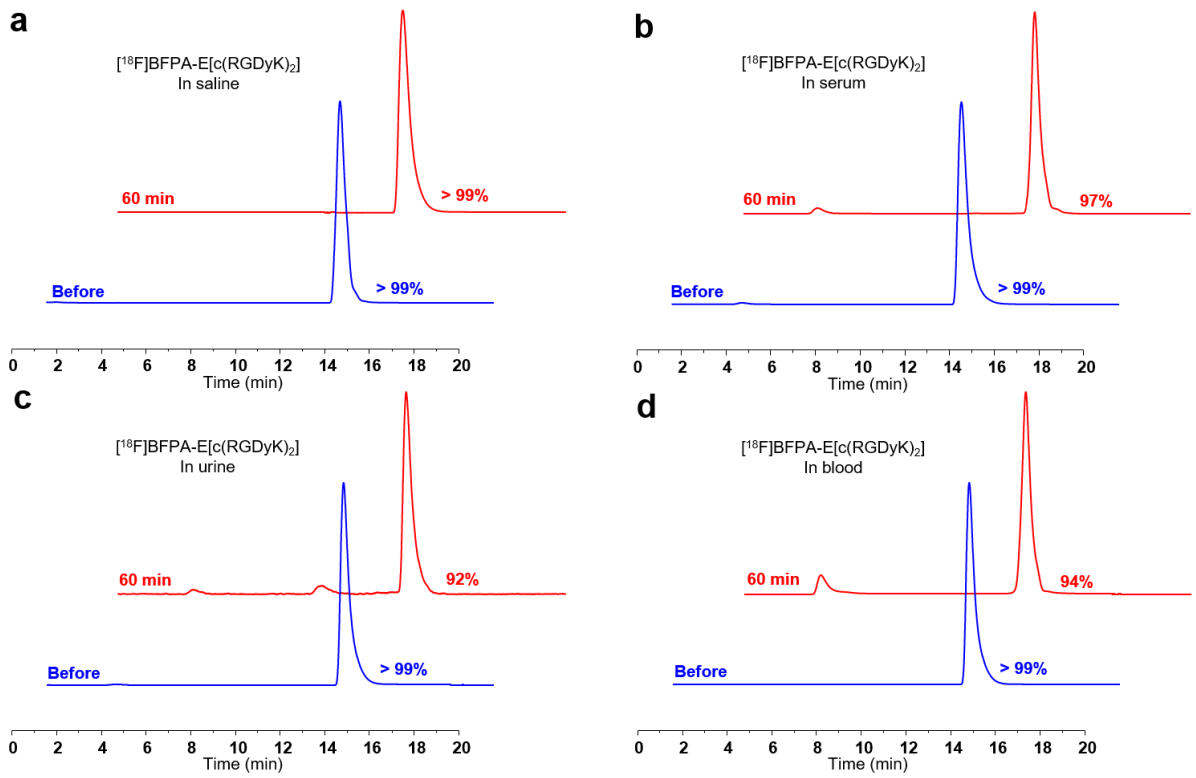
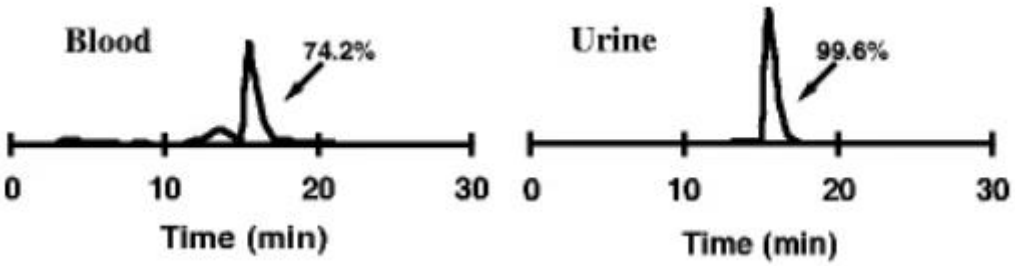


Figure S35. *In vitro* and *in vivo* stability of [^{18}F]BFPA-E[c(RGDyK)]$_2$. (**a**, **b**) *In vitro* stability of [^{18}F]BFPA-E[c(RGDyK)]$_2$ in saline and serum. Radio-HPLC analysis demonstrated that the stability of [^{18}F]BFPA-E[c(RGDyK)]$_2$ in saline > 99% and serum > 97% *in vitro*. (**c**, **d**) *In vivo* stability of [^{18}F]BFPA-E[c(RGDyK)]$_2$ at 60 min post *i.v.* injection. Radio-HPLC analysis demonstrated that [^{18}F]BFPA-E[c(RGDyK)]$_2$ remains stable at over 92% in urine and over 94% in blood *in vivo*.



Cited Figure From: Metabolic stability of ^{18}F-FPRGD2 in mouse blood and urine samples at 1 h after injection. *Eur J Nucl Med Mol Imaging* **34**, 1823–1831 (2007), https://doi.org/10.1007/s00259-007-0427-0.

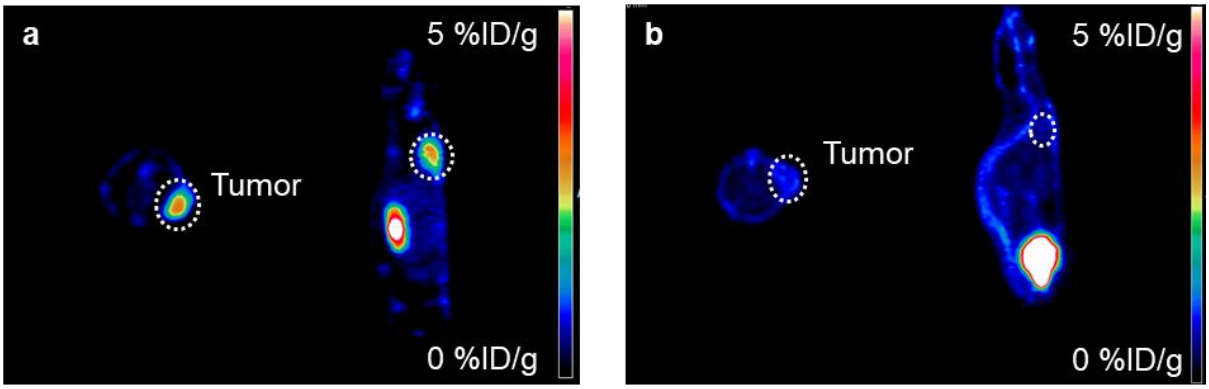


Figure S38. **a** MicroPET images of [^{18}F]BFPA-E[c(RGDyK)]$_2$ in U87MG xenograft mice at 30 min after tail vein injection. **b** MicroPET images of U87MG xenograft mice at 30 min after simultaneous injection of [^{18}F]BFPA-E[c(RGDyK)]$_2$ and E[c(RGDyK)]$_2$ (200 μg).

3) The mechanistic studies, while informative, are not fully conclusive. Isolation and more complete characterization of the proposed phosphonium intermediates would provide stronger support for the proposed mechanism. Computational studies alone are not sufficient.

Re: We appreciate your suggestion regarding the isolation and characterization of the proposed phosphonium intermediates.

As mentioned in our manuscript, we have indeed made multiple attempts to isolate the proposed phosphonium intermediates; however, these efforts have not yet yielded successful results. We believe that the inherent instability of the intermediates under the reaction conditions may contribute to this challenge. Despite these limitations, we have added new evidence in the new **Figure 3** to provide further evidence supporting our proposed pathway. **Figure 3a** outlines the key bond formation and cleavage steps involved in the fluorination process. In the new **Figure 3b**, we present *in situ* ^{31}P NMR spectra of the reaction mixture (included NMR—specifically ^{1}H NMR, ^{31}P NMR, and ^{19}F NMR spectra in **Figure S39**–**S42**), offering direct mechanistic insights. Additionally, through control experiments (the new **Figure 3c**), we have excluded the possibility of certain intermediates. Finally, in the new **Figure 3d**, MS analysis reveals the detection of plausible intermediates, further substantiating the proposed mechanism (MS data are shown in **Figure S43**).

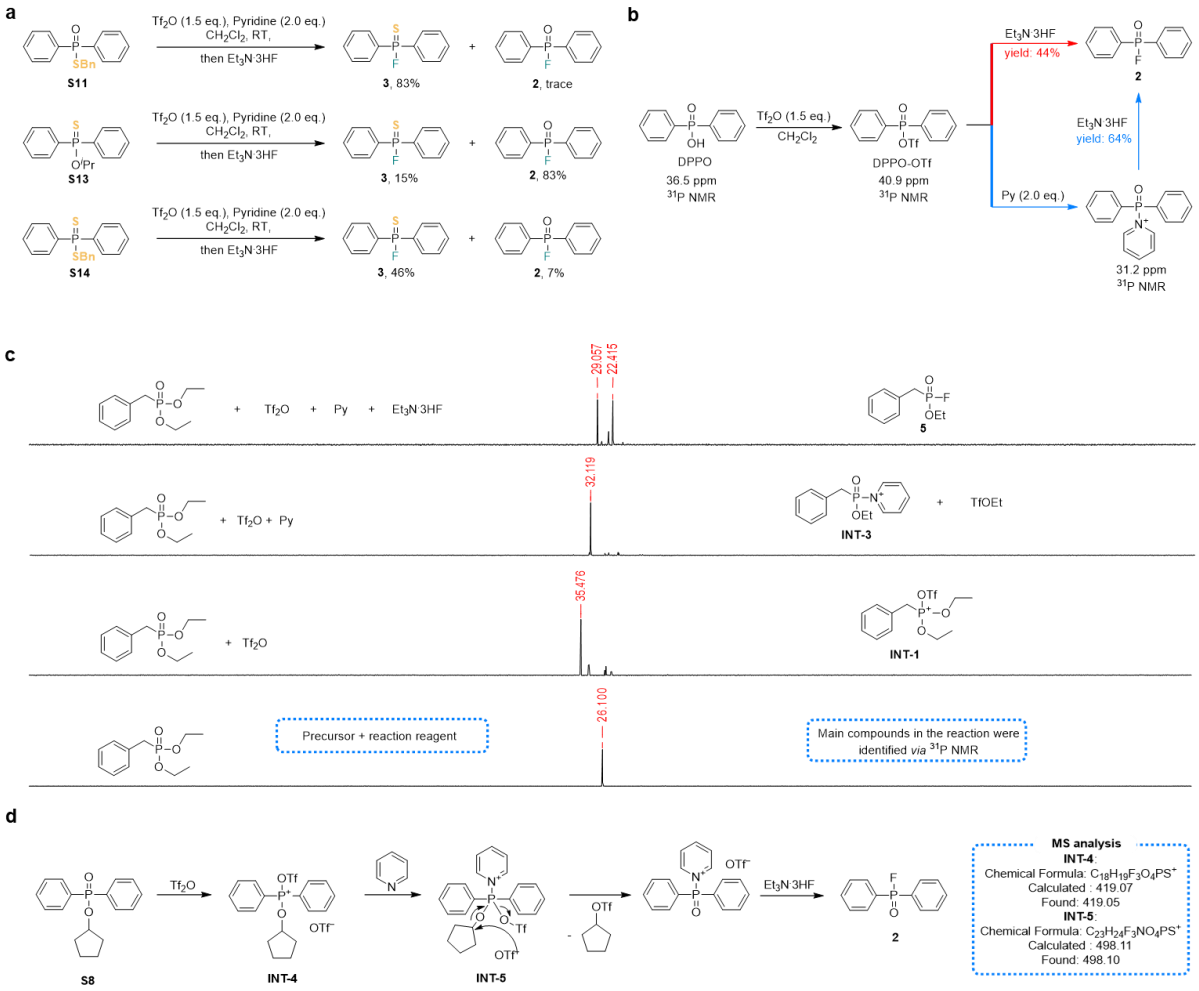


Figure 3. **The mechanism study of late-stage fluorination of alkyl phosphonates *via* electrophilic activation. a**, Control experiment on the direct fluorination of thiophosphonic alkyl ester. **b**, ^{31}P NMR monitoring of stepwise reaction intermediates *in situ*. **c**, Control experiment of pyridine with DPPO as precursor. **d**, Stepwise *in situ* mass spectrometric monitoring of fluorination using precursor compound **S8**.

4) The manuscript would benefit from more context on how this method compares to and improves upon existing late-stage ^{18}F-fluorination approaches for alkyl phosphonates and other substrates. A more thorough discussion of advantages, limitations and scope is recommended.

Re: Thanks for this constructive comment. We have included a more comprehensive comparison in the revised manuscript for this very first approach to achieve late-stage nucleophilic ^{18}F-labeling of alkyl phosphonates, as demonstrated in **Figure 1a**.

1. The following statement has been added to the "**Introduction**" section of the revised

manuscript. "The current direct ^{18}F-labeling through nucleophilic substitution of aryl phosphonate precursors, aimed at overcoming the low molar activity (A_m) associated with isotope exchange-based ^{18}F-labeling method produces ionic ^{18}F-fluorophosphonates that hinder cell uptake and exhibit high bone affinity, limiting their bioavailability."

2. The following statement has been added to the "**Late-stage ^{18}F-fluorination of ^{18}F-tracers**" section of the revised manuscript. "While ^{18}F/^{19}F-isotope exchange facilitates direct ^{18}F-labeling of highly functionalized peptides in one step, a significant limitation is the inseparability of the precursors from the ^{18}F-product, resulting in an A_m of only 2.22–4.81 GBq/μmol. The labeling method described in this study demonstrates the potential to enhance the A_m by nearly 100-fold (251 ± 12 GBq/μmol)."

5) Some additional controls should be included, such as ^{18}F-labeling of non-alkyl phosphonate precursors, to confirm selectivity and functional group tolerance.

Re: Thanks for this constructive comment. The following controls have been included and discussed in the revised manuscript. “This selectivity was further confirmed by the ^{18}F-fluorination of a mixed phosphonate precursor (**S15**) substituted with −OEt and −OPh groups (see **Figure S1** for details).”.

We conducted ^{18}F-labeling experiments using a −OEt and −OPh substituted mixed phosphonate (**S15**) as the precursor. **S15** undergoes highly selective ^{18}F-fluorination, yielding an unstable phenyl benzylphosphonofluoridate, which, consistent with literature reports (*Org. Lett.*, **23**, 11, 4261–4266, (2021), https://doi.org/10.1021/acs.orglett.1c01211), is prone to hydrolysis and cannot be isolated due to its instability, spontaneously converting into benzylphosphonofluoridic acid. The results confirmed the high selectivity for alkyl phosphonate esters and demonstrated the tolerance for phenyl esters, while also supporting our proposed mechanism. The detailed descriptions and HPLC traces have been added in **Figure S2**.

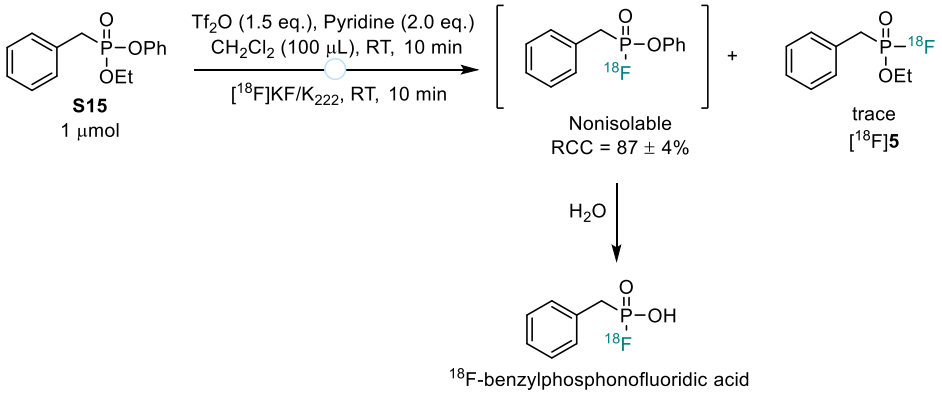


Figure S1. The selectivity of the reaction was verified by ^{18}F-fluorination.

6) It's unclear if this radiofluorination approach works for more complex biologically relevant molecules beyond the few examples shown.

Re: This point is well taken. Due to the inherent electrophilicity of Tf_2O, functionalities such as hydroxyl, carboxyl, and amino groups present significant challenges (*Nature* **594**, 217–222 (2021), https://doi.org/10.1038/s41586-021-03567-3) during direct radiofluorination. To successfully label molecules containing such incompatible functional groups—especially larger targets like peptides—we have developed a two-step ^{18}F-labeling approach. As illustrated in **Figure 6b** of the revised manuscript, we utilize activated esters to achieve the ^{18}F-labeling of these complex compounds. Our existing findings demonstrate that, even in the presence of challenging functional groups, radiofluorination of biologically relevant molecules can be effectively performed.

In the result section, under "**Late-stage ^{18}F-fluorination of ^{18}F-synthons**" subtitle, we have also included the following content to elaborate on the scope of this labeling method: "When the precursor structure contains electron-rich functional groups—such as amino, carboxyl, hydroxyl, or amide groups—that are incompatible with electrophilic activation conditions, a two-step strategy can be employed to achieve ^{18}F-labeling through [^{18}F]BFPA."

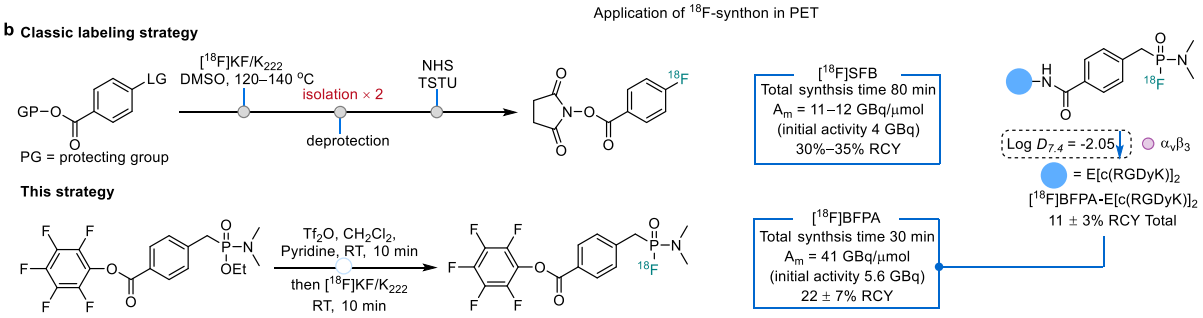


Figure 6b. Preparation $\alpha_v\beta_3$ integrin receptor developer [^{18}F]BFPA-E[c(RGDyK)]$_2$.

7) The manuscript would benefit from more quantitative discussion of how this method improves upon existing approaches in terms of radiochemical efficiency, synthesis time, automation capability etc.

Re: Thank you for your constructive feedback.We have already included a quantitative discussion of A_m in response to *the question 4)* in the revised manuscript. Additionally, we have expanded the discussion in the revised manuscript's "**Late-stage ^{18}F-fluorination of ^{18}F-tracers**" section to include the following: "Employing an automated synthesis module enables the radiosynthesis of the chloromethyl derivative [^{18}F]**42**, which is tailored to target DNA guanine, to proceed from an initial activity of 11.2 GBq. This approach significantly improves

upon traditional manual labeling methods, particularly when using low starting activities. The automated process allows for the use of higher starting activities, leading to an approximately tenfold increase in A_m (251 ± 12 GBq/μmol, n = 3), compared to manual methods. Additionally, the RCY achieved is 34 ± 7%, with the entire synthesis being completed in a considerably shorter time frame. The automation not only enhances efficiency but also improves reproducibility, making it a superior approach in radiochemical synthesis (Section **5** of the Supplementary Information)."

Reviewer #2

This is a highly interesting work that develops a novel method for synthesizing organic fluorophosphines. Through electrophilic activation by Tf_2O and optimized conditions, the reaction completes within 15 minutes and achieves a yield of 99%, making it more efficient and convenient than traditional strategies. Furthermore, the team applied this new methodology to the late-stage radiofluorination of a broad range of dialkyl and monoalkyl phosphonates, and they tested it in vivo. Overall, the presented work is of significant interest, well-executed, and I recommend accepting this paper for publication. However, there are a few concerns and questions that, if addressed, would further solidify the authors' work:

Re: Thanks for the positive comments. Comprehensive revisions have been made to address the constructive concerns and questions.

1) The limitations of prior work presented in Fig. 1a are confusing and should be clarified. What do the blue and red circles represent, and why are they considered limitations of the previous work?

Re: **Figure 1a** has been revised carefully. It has been stated in the legend that the blue and pink balls represent different substituents attached to the phosphorus atom (P). The limitations of previous work involve the reliance on unstable intermediates, strong acids/bases reflux, and explosive reagents. In the revised manuscript, we have added illustrations in **Figure 1a** to clearly indicate the harsh conditions associated with strong acids and bases, as well as the use of explosive reagents. Achieving late-stage fluorination also enhances the atomic economy of our method.

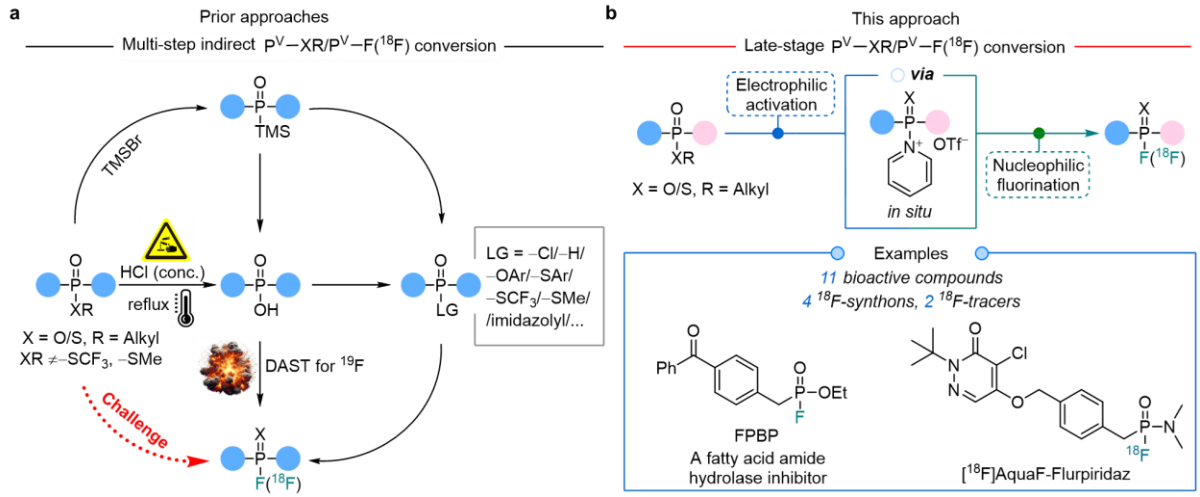


Figure 1. Approaches for fluorination of alkyl phosphonates. a, Prior approaches rely on multi-step conversion from symmetrical alkyl phosphonates and isolation of intermediates. **b**, This late-stage approach from alkyl phosphonates eliminates the requirement for hash conditions, intricate multi-step transformations and separation. OTf^-, trifluoromethanesulfonate; TMSBr, bromotrimethylsilane; DAST, diethylaminosulfur trifluoride; LG, leaving group; Ar, aryl; Me, methyl. The blue and pink balls represent different substituents.

*2) When designing and synthesizing alkyl phosphonate precursors for ^{18}F-tracers, it is crucial to preserve the core activity or function of the original molecules. The compounds [^{18}F]**31a-35a** lack characterization after the addition of the organic fluorophosphine moiety. To strengthen the radiofluorination strategy, please provide the cLog P and other drug property parameters of the original molecules and the ^{18}F-tracers. Additionally, it would be beneficial to include the binding affinity of both the original molecules and the ^{18}F-tracers to their target proteins.*

Re: Thanks for the constructive comments.

cLog *P* Values: Log $D_{7.4}$ values were determined for the two PET tracers, [^{18}F]BFPA-Flurpiridaz ([^{18}F]**45**) and [^{18}F]BFPA-E[c(RGDyK)]$_2$, along with the corresponding log *P* values for the original tracers [^{18}F]Flurpiridaz (Circulation **119**, 2333– 2342 (2009), https://doi.org/10.1161/CIRCULATIONAHA.109.854919) and ^{18}F-FPRGD2 (*Eur J Nucl Med Mol Imaging* **34**, 1823–1831, (2007), https://doi.org/10.1007/s00259-007-0427-0).

Theoretical predicted cLog *P* values for compounds **40**–**44** and their corresponding original molecules were also included. The Log $D_{7.4}$ or cLog *P* values for [^{18}F]**40**–[^{18}F]**45** and [^{18}F]BFPA-E[c(RGDyK)]$_2$ have been incorporated into the revised manuscript in **Figure 6**, while the corresponding original molecules' values are provided in **Table S21** of the revised Supplementary Information. From the comparison, it was found that compounds modified with fluorophosphine moiety exhibit a decreasing trend in Log $D_{7.4}$ or cLog *P* in some cases, indicating changes in lipophilicity and potential pharmacokinetic properties. (cLog *P* values were predicted using ALOGPS 2.1 http://www.vcclab.org/lab/alogps).

Binding Affinity: We experimentally determined the IC_{50} value for the proof-of-concept tracer [^{18}F]BFPA-Flurpiridaz, which demonstrates promising clinical potential for PET imaging of myocardial ischemia. The IC_{50} value for [^{18}F]BFPA-Flurpiridaz was found to be 148.0 nM, which is significantly lower than the IC_{50} of 248.2 nM for the parent compound Flurpiridaz. This suggests that the modified with fluorophosphine moiety tracer exhibits enhanced binding

affinity.

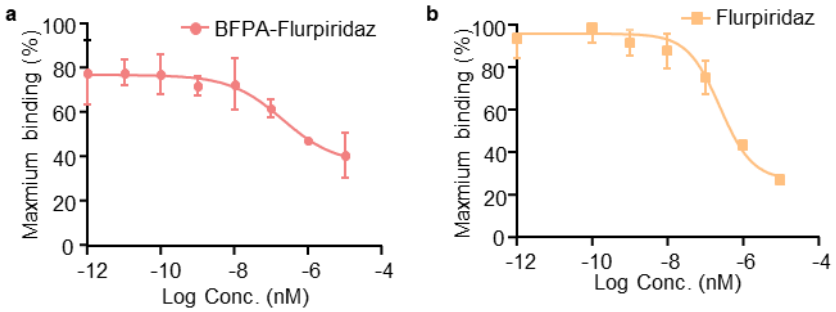
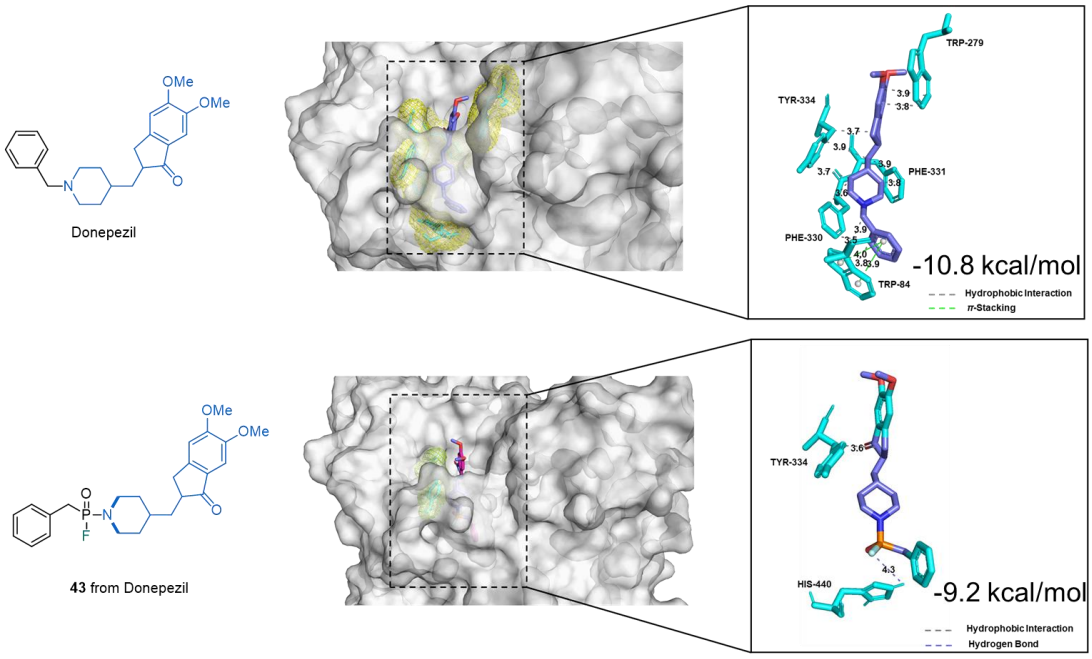


Figure S36. IC_{50} curves of BFPA-Flurpiridaz and Flurpiridaz inhibiting [^{18}F]Flurpiridaz.

Besides, the molecular docking simulations compared the binding affinity of the original molecules and the ^{18}F-tracers to their target proteins, offering neglectable affinity changes or enhanced binding due to the hydrogen bonding interactions provided by the P═O double bond of the building block with specific residues on the protein, as illustrated in **Figures S37c**, **Figures S37d**, **Figures S37e**.

c. Docking Donepezil and **43** to AChE. (PDB: 1EVE)



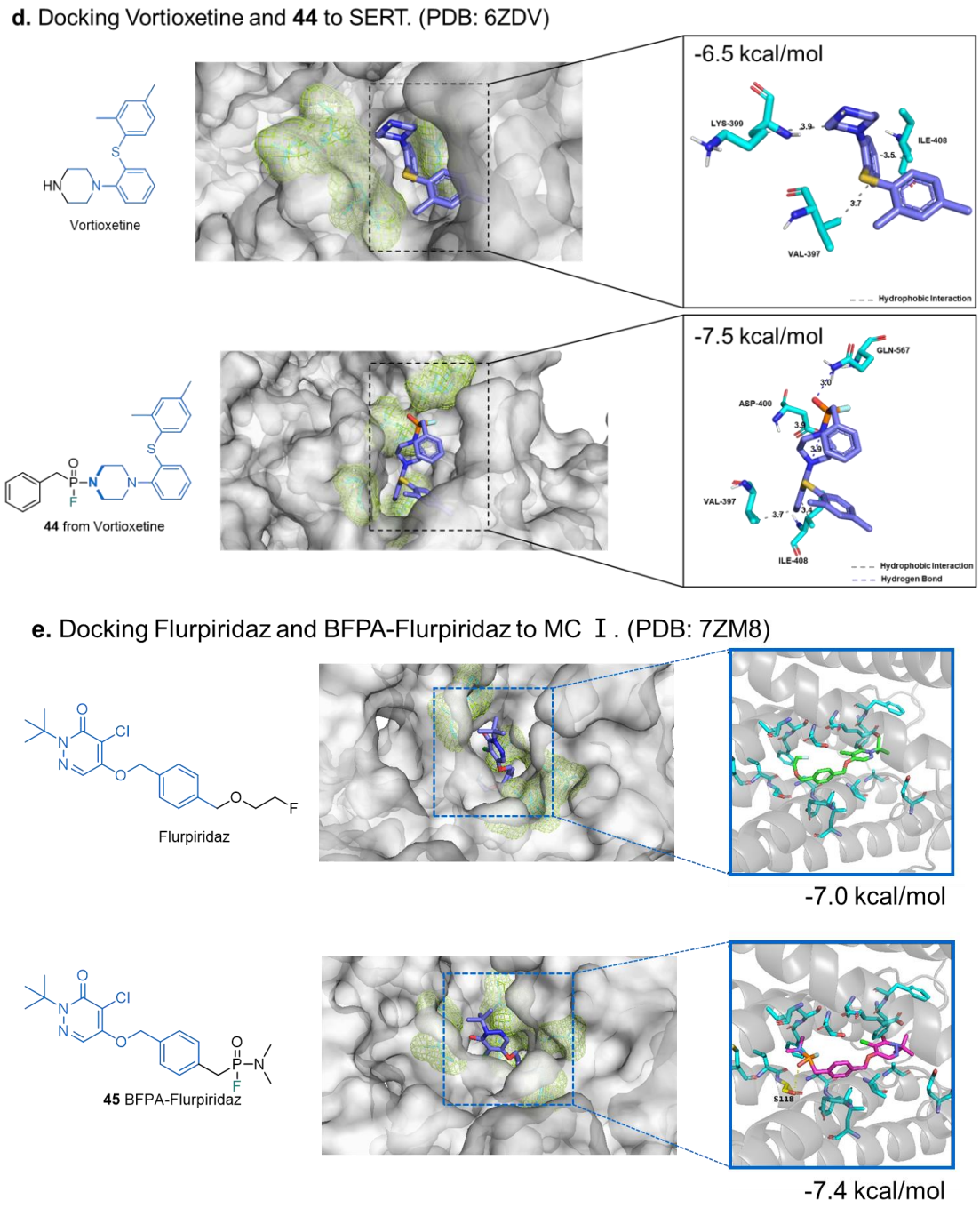


Figure S37. Ligand−protein docking. All protein structures are sourced from the Protein Data Bank (PDB, http://www.rcsb.org/).

3) The authors claim that P-benzyl fluorophosphonamide exhibits excellent stability under both in vitro and in vivo conditions. Please provide the stability data for both in vitro and in vivo conditions to support this claim.

Re: Thanks for the constructive comments. Please see the reply to *the question 2) of Reviewer 1*.

Reviewer #3

The manuscript entitled "Late-Stage (Radio)Fluorination of Alkyl Phosphonates via Electrophilic Activation", authored by Zhang et al., describes a method to make alkyl fluorophophonates from activated phosphinates in situ, extending its application to ^{18}F-radiochemistry. This non-radioactive approach as well as radioactive version to fluorinate phosphorus containing moiety in organic entities would be useful for many purposes, as significant level of investigation has been made. Although the reported procedure may find useful application, the substrate scope to reflect electronic nature and steric, apart from complexity and numbers of substrate employed, in this study seems insufficient for applications in organic community in general. As an example, instead of adopting similar electronic nature of substituent as described in Fig 4. (a) (para-pattern, halogens, EWG), the inclusion of one or two meta-EWG, EDG and ortho-EWG, EDG would be beneficial to readers interested in this methodology. Therefore, it is strongly suggested to implement additional substrates (with diverse substitution patterns and electronics) to prove the generality of proposed method, ideally with one or two examples of highly functionalized substrates. Refer to other points described below for improvement. Some aspects of the work should be explained in greater detail in both main text and supporting materials. After those have been fully addressed, I would expect this article to meet the stringent publication criteria of Nature Communications. I would suggest improving the manuscript before acceptance.

Re: Thank you for your constructive suggestions. In the revised manuscript, as shown in the new **Figure 5a**, the substrate scope has been expanded according to your recommendations. Both *ortho*- and *meta*-substituted substrates with varying electronic properties have been incorporated, including a *meta*-EDG (**14**), *meta*-EWG (**16**, **17**), and *ortho*-EWG (**19**, **20**, **21**). Additionally, three highly functionalized substrates derived from FPND derivate (**26**), Flurpiridaz (**27**), and Socticlestat (**28**) have been synthesized. Excellent tolerance of these highly functionalized and medically significant substrates has been observed, demonstrating the robustness and broad applicability of the method.

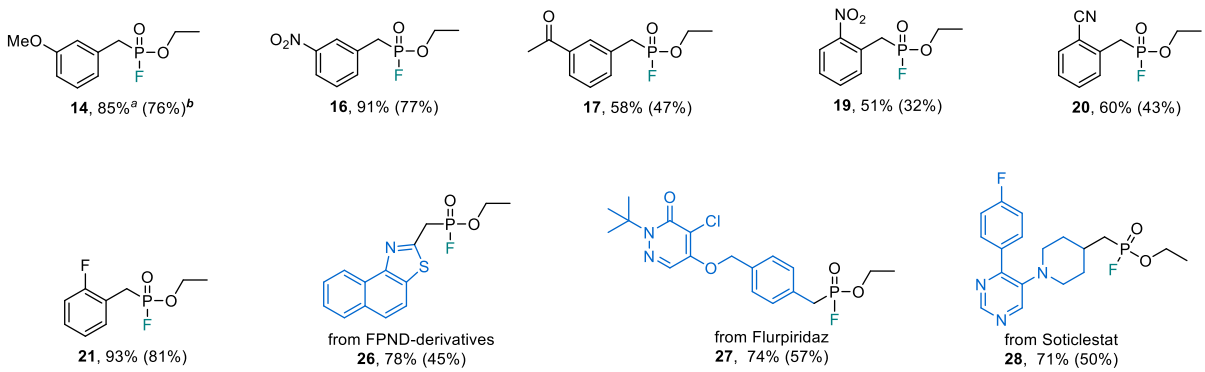


Figure. Enlarged substrate scope for late-stage fluorination of alkyl phosphonates *via* electrophilic activation. [a]Conversions determined by ^{31}P NMR. [b]isolated yield.

Points to improve manuscript:

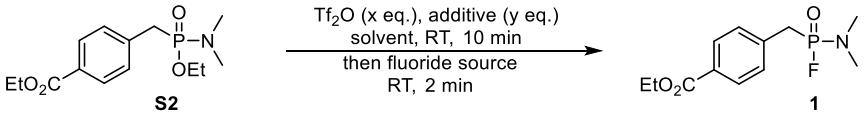
1) Since electrophilic activation appears to be a crucial step in this transformation and the rationale behind this activation is referenced, it is unclear why Tf_2O is used to activate the dialkyl phosphonate. Is the 1.5 equivalent of Tf_2O necessary for a successful reaction? Unlike the non-radioactive fluorophosphonate synthesis, it is important to avoid using excess activator in the production of ^{18}F-radiotracers to simplify the downstream quality control workload (such as chemical and radiochemical purity).

Re: Thanks for this constructive comment.

1. The screening results of electrophilic activators have presented in **Table S1** of the revised supplementary information. Classic activators such as trifluoromethanesulfonic anhydride (Tf_2O, entry 8) and trifluoroacetic anhydride (TFAA, entry 9) were tested for dialkyl phosphonate activation, with Tf_2O demonstrating excellently superior performance. Additionally, "–OTf" has a Hammett substituent constant of 0.84 (*J. Org. Chem*. **41**, 781–785 (1976), https://doi.org/10.1021/jo00867a007), making it one of the strongest electron-withdrawing groups, comparable to "$–N(CH_3)_3^+$", and an excellent leaving group. The strong electrophilicity of Tf_2O enables it to activate electron-rich systems, such as amides, carbonyls, sulfonate esters, and phosphonate esters, generating OTf-substituted intermediates for further transformations. Our research uniquely applies Tf_2O to mediate electrophilic activation in the late-stage (radio)fluorination of alkyl phosphonates, addressing specific challenges and demonstrating novel reactivity patterns.
2. In **Table S1**, we screened the amounts of Tf_2O and pyridine. At 1.0 eq. of Tf_2O, the precursor was not fully consumed, while 2.0 eq. led to overreaction and byproducts. Typically, the yield is optimal when the amount of base used exceeds that of Tf_2O.

3. For ^{18}F-labeling, 1.5 eq. of Tf_2O to the precursor was found to be quite successful. Water quenching and C18 cartridge pre-purification effectively removed water-soluble impurities, including excess Tf_2O, simplifying quality control.

Table S1. Selected optimization table for alkyl phosphonates fluorination[a]



Entry	Tf_2O/x eq.	Additive/ y eq.	Solvnet[b]	Fluoride source	Conversion (%)[c]
1	1.0	Pyridine/1.0	CH_2Cl_2	TBAF	11 ± 6
2	1.0	Pyridine/1.5	CH_2Cl_2	TBAF	36 ± 3
3	1.5	Pyridine/1.0	CH_2Cl_2	TBAF	6 ± 2
4	2.0	Pyridine/1.5	CH_2Cl_2	TBAF	19 ± 3
5	2.0	Pyridine/2.0	CH_2Cl_2	TBAF	27 ± 5
6	1.5	Pyridine/2.0	CH_2Cl_2	TBAF	47 ± 1
7	1.5	Diphenylsulfane/2.0	CH_2Cl_2	TBAF	0
8	1.5	Pyridine/2.0	CH_2Cl_2	$Et_3N{\cdot}3HF$	93 ± 3
9[d]	TFAA, 1.5 eq.	Pyridine/2.0	CH_2Cl_2	$Et_3N{\cdot}3HF$	trace
10	1.5	Pyridine/2.0	THF	$Et_3N{\cdot}3HF$	trace
11	1.5	Pyridine/2.0	Toluene	$Et_3N{\cdot}3HF$	11 ± 2
12	1.5	Pyridine/2.0	CH_3CN	$Et_3N{\cdot}3HF$	35 ± 2
13	1.5	Pyridine/2.0	1,4-Dioxane	$Et_3N{\cdot}3HF$	3 ± 1

[a] Reactions were performed using 0.2 mmol **16**, tetrabutylammonium fluoride (TBAF 1.2 eq.)/$Et_3N{\cdot}3HF$ (0.5 eq.) in solvent (0.2 M).

[b] CH_2Cl_2: dichloromethane; THF: tetrahydrofuran; CH_3CN: acetonitrile.

[c] Cinversions determined by ^{31}P NMR. Replace Tf_2O with trifluoroacetic anhydride (TFAA).

2) The reason for using an N-heteroaromatic base was not fully explained. Have common bases like Et_3N or DIPEA been considered for this purpose? What reasons exist for not including these bases in this transformation?

Re: Thanks for this constructive comment. In the revised manuscript, an evaluation of common bases including DBU, Et_3N, DIPEA, and K_2CO_3 was conducted, with the results now presented

in the updated **Figure 2a**. The new description has been added in the revised manuscript (page 4): "In addition to *N*-heteroaromatic bases, several common bases were also evaluated. Et_3N (**a**^{8}) and DIPEA (**a**^{9}) were less effective due to steric hindrance, while K_2CO_3 performed poorly because of its low solubility in CH_2Cl_2. In contrast, DBU yielded 33%."

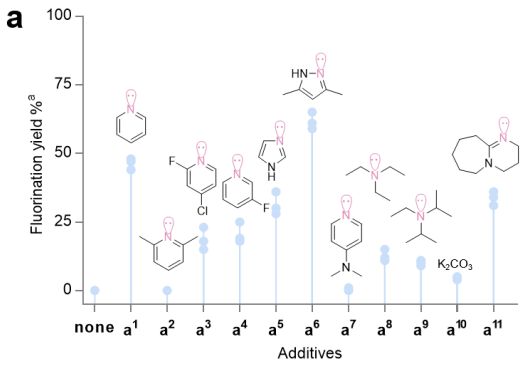


Figure 2. Optimization of conditions for late-stage (radio)fluorination of alkyl phosphonates *via* electrophilic activation. a, Optimization of *N*-heteroaromatic bases, ethyl 4-(((dimethylamino)(ethoxy)phosphoryl)methyl)benzoate (**S2**) as a model compound.

3) In this transformation, the fluoride source was identified as Et_3N3HF and used throughout the experiments. In ^{18}F-labeling, the ^{18}F-K_{222}/K_2CO_3 complex was utilized as the ^{18}F-fluorine source. Since the nonradioactive modeling reaction was performed on a small scale (i.e., 0.2 mmol) and characterized with NMR, it would be beneficial to demonstrate the KF/cryptand combination (a similar nucleophilic fluoride source as used in radiochemistry) to improve nucleophilicity and solubility in organic solvents.

Re: Thanks for the comment. The K_{222}/KF has been newly employed as a candidate fluoride source for the ^{19}F-fluorination, as shown in the new **Figure 1b**. However, the fluorination yield of only 17% was rather low compared to using $Et_3N{\cdot}3HF$ as the fluoride source (fluorination yield 92%). In ^{18}F-fluorination, the screening results of fluorine sources are presented in **Table S3** of the revised supplementary information, where [^{18}F]KF/K_{222} exhibited superior performance. Notably, [^{18}F]$Et_3N{\cdot}3HF$ was less commonly used for ^{18}F-fluorination.

4) In certain cases, ^{31}P NMR was utilized to determine the chemical yield of a reaction. It is recommended to include specific experimental details for accurate yield calculation with ^{31}P qNMR. It is common practice to assess chemical yield using heteronucleus like ^{19}F. Apart from the natural abundance of each nucleus, differences in relaxation time and delay parameters between ^{19}F and ^{31}P NMR could potentially lead to varying quantitative NMR results.

Re: Thanks for this constructive comment.

1. The specific experimental details for ^{31}P qNMR have been included in the revised **Figure 2** caption. In consistence with the internal standard quantification principles, ^{31}P qNMR was conducted by adding CD_2Cl_2 after the reaction, with the preliminary conversion calculated from the ratio of product to byproduct peak areas. The difference between the isolated yield and ^{31}P qNMR yield attributes to losses during purification and potential instability of the product.
2. The yield not detected by ^{19}F qNMR stems from the complexity of the reaction mixture, which contains various fluorine-containing species and byproducts, such as Tf_2O, TfOEt, OTf^-, and F^-. These compounds produce overlapping signals, with peak areas much larger than those of the desired fluorinated product, making accurate quantification of the ^{19}F signals challenging.

5) In proposing a mechanism with computational observation (Figure 3), the oxidation state at the phosphorus center might influence the overall chemical intermediacy and reaction pathway. Can you propose a relationship between the oxidation state of central phosphorus and the outcomes of electrophilic activation leading to final product (such as TS-1, INT-1, INT-2) in terms of stability, geometry, and reactivity? This could be very helpful to support the proposed mechanism rather than just showing the reaction progressing path.

Re: Thanks for this constructive comment. To clarify the relationship between the oxidation state of central phosphorus and the proposed mechanism, we have introduced a less likely alternative pathway in the revised Supplementary Information (**Figure S46**). This figure systematically illustrates the structures associated with different phosphorus oxidation states and their potential impact on reaction pathways.

In our discussion, we have also addressed the presence of the OTf-substituted λ5σ5-type intermediate **INT-S2**. We noted that "Given that the intermediate **INT-1** cannot react with $Et_3N{\cdot}3HF$ to yield the desired fluorinated product without Py, the λ5σ5-type OTf-substituted intermediate **INT-S2** is excluded," which has been incorporated into the revised manuscript.

Additionally, we further explored the relationship between different oxidation states of phosphorus intermediates and the departure of the ethyl fragment. We stated: "Formation of **INT-3** proceeds through two possible pathways for the departure of the ethyl fragment via Arbuzov-like processes. One route involves direct nucleophilic attack by Py on the methylene

of the ester in **INT-1**, yielding the 1-ethylpyridinium byproduct with an activation barrier of 16.7 kcal/mol (**Figure S46**). Another pathway involves initial interaction between Py and **INT-1** to form **INT-2**, followed by OTf^- attacking the methylene of the ester in **INT-2**, leading to the departure of TfOEt and the formation of **INT-3**, which requires a lower activation barrier of 12.9 kcal/mol. The latter pathway is energetically favored."

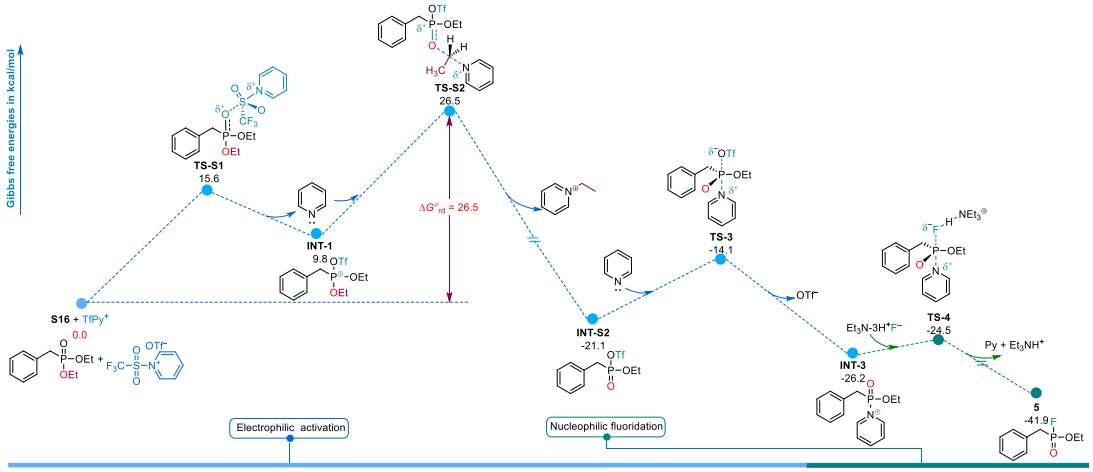


Figure S46. The energy distribution of alkyl phosphonate (**S16**) activated by Tf_2O for fluorination at a less likely alternative pathway.

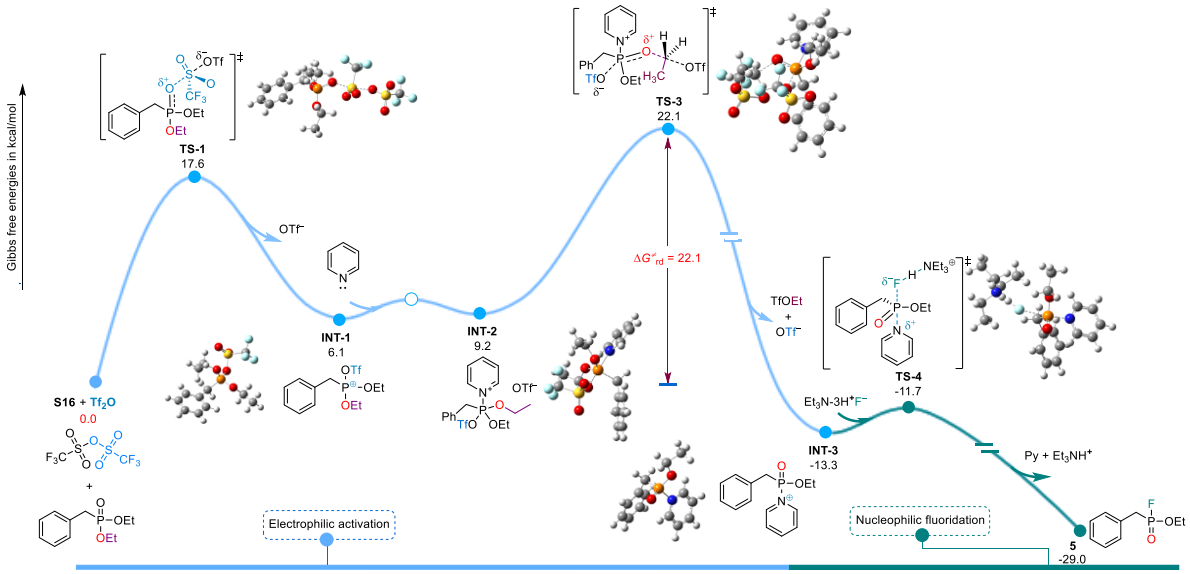


Figure 4. The energy distribution of alkyl phosphonate (**S16**) activated by Tf_2O for fluorination and the optimal structure of key stability points were investigated using DFT calculations. The calculated Gibbs energy (ΔG and $\Delta G_{rd}^{\neq}$, 298.15 K, 1.0 atm) is expressed in kcal/mol. For detailed configuration, see the Supplementary Information (8.2−8.3).

6) In Figure 1, (a), readers may mistakenly think that ^{18}F-labeled alkyl phosphates come from ^{18}F-DAST, even though it is mentioned in parentheses, which is an electrophilic radiofluorination. This requires clarification.

Re: Thanks for the comment. We have added a note in the new **Figure 1a** indicating "DAST for ^{19}F" to clarify that the ^{18}F-labeled alkyl phosphates are not directly produced by DAST.

7) Throughout the manuscript, the compound numbering system needs to be more organized. Preferably with brackets and other symbols or alphabets. Such as 1b, rac-1a, INT-1' (should be in Figure 3(b)), and many more are not properly described in the main text and have caused confusion. Coordinate compound numbering with the supporting information properly as well.

Re: Thanks for this comment. In the revised manuscript and Supplementary Material, we have systematically renumbered all compounds mentioned. All phosphonate fluorination precursors are now numbered as **S1**–**S57**, and the resulting fluorinated phosphonates are numbered **1**–**46**, with '[^{18}F]' added before the corresponding Arabic numerals to indicate the ^{18}F-products. Intermediates in the synthesis of phosphonate precursors are labeled as **i-1** to **i-10**, and unstable intermediates in mechanistic studies are represented as **INT-1** to **INT-5**." The bases in the additive screening are represented by the letter '**a**' and superscript Arabic numerals 1–11 (**a^{1}**-**a^{11}**).

8) ^{31}P NMR in Figure 3 (b) is very hard to read. It would be nice to redraw the figure in order to be read easily (especially chemical shift in ppm).

Re: Thanks for the comment. The ^{31}P NMR has been enlarged and placed in the new **Figure 3c** to improve readability.

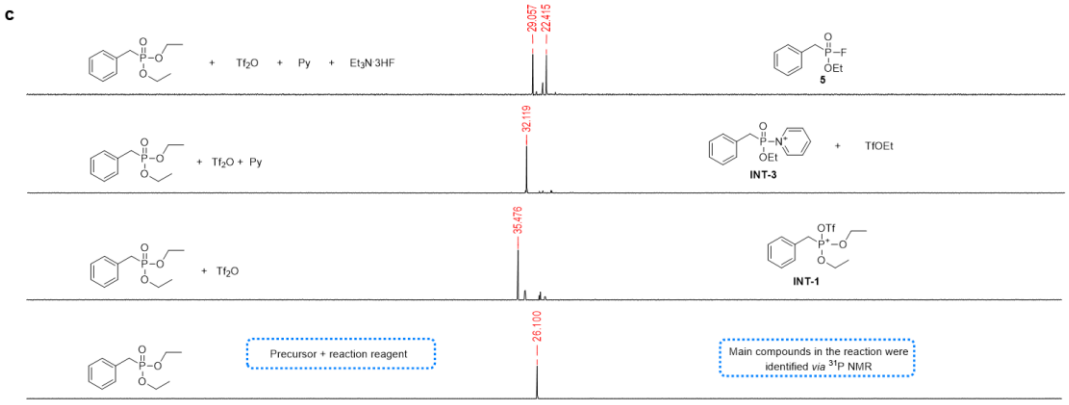


Figure 3c, ^{31}P NMR monitoring of stepwise reaction intermediates *in situ*.

9) Authors are advised to adhere to the guidelines related to radiochemical notation. The terms "RCC" and "RCY" are confusing. Refer to the guideline and modify the expression accordingly (see, Nucl Med Biol 2017, 55, v; ibid.). 2019, 71, 19; ibid. 2021, 93, 19).

Re: Thanks for pointing out the issues related to radiochemical notation.

We have reviewed all figures and text to ensure that the terminology aligns with the latest naming guidelines (*Nucl. Med. Biol.* **93**, 19–21 (2021), https://doi.org/10.1016/j.nucmedbio.2020.11.003). In the revised manuscript, we have included the statement "RCCs determined by radio-TLC (n = 3) are denoted as RCC_{TLC}. RCY = isolated ^{18}F-product activity amount (decay corrected) / starting amount of radioactivity " in the caption of **Figure 6**.

Additionally, in the Supplementary Information of the **4. Experimental Procedures for Radiochemistry**, appropriate clarifications regarding RCC and RCY have been made in accordance with the new guidelines. Specifically, "radiochemical conversion (RCC) was assessed using radio-TLC by dividing the area under the curve (AUC) of the radioactive peak of interest by the total AUC of all radioactive peaks. The radiochemical yield (RCY) is based on the ratio of the final isolated product to that of the initial radioactivity, with all amounts decay corrected to the same time."

*10) In Supplementary Materials S17: There was no solvent used to make **1**. Is this originally solvent-free reaction? Please specify.*

Re: Thanks for the comment. This is a solvent-free Arbuzov reaction with alkyl halides and triethyl phosphite. A note has been added in the revised experimental methods section of the revised Supplementary Information.

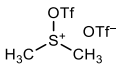
11) In Supplementary Materials S10: It would be helpful to include the mixed base stoichiometry for experimental details. Additionally, in S14, the ratio between alcohol and thiol should be clearly specified.

Re: Thanks for the comment. We apologize for any confusion regarding the use of mixed bases. The correct description is as follows has been incorporated into the Supplementary Information.: "A mixture of RXH and base (base = 2.0 eq. Et_3N for R = Ar/Bn; base = 1.2 eq. NaH for R = Alkyl) was gradually added dropwise under an ice bath,"

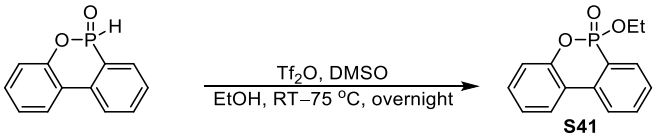
12) In Supplementary Materials S9: Is there a specific reason to track the reaction progress using ^{31}P NMR? Can we monitor the reaction progress effectively using traditional TLC instead?

Re: Thanks for the comment. TLC has certain limitations for unstable intermediates, as products (phosphorochloridate) may hydrolyze on the basic TLC silica gel and obscure results. In contrast, using ^{31}P NMR allows for easy identification of products through chemical shift and quantification of conversion *via* peak areas. In the revised Supplementary Materials, it was introduced that "While TLC is ineffective in detecting the reaction process of unstable intermediates, whereas ^{31}P NMR allows for clear product identification and quantification of conversion."

13) In Supplementary Materials S15, what is the role of DMSO? The scheme in S15 is misleading because it appears to depict DMSO as a solvent.

Re: Thanks for the comment. DMSO has been placed above the reaction arrow in the scheme to clarify that it is not a solvent. EtOH, which functions as both a reactant and solvent, is positioned below. Additionally, the revised Supplementary Materials now includes: "Tf_2O and DMSO form an *in situ*  active intermediate to initiate the reaction" to explain DMSO's role.

Procedure J for the formation of S41



14) In the Supplementary Materials S46, it is generally known that Cs_2CO_3 has very limited solubility in neat MeCN (a relatively large amount of Cs_2CO_3 is used for 100 μL of MeCN). Does this impact the overall RCC as indicated in Table S3, entry 2?

Re: Thanks for the comment. After the reaction was completed, 9900 μL of water was added to quench it. The reaction RCC_{TLC}s were then determined using radio-TLC with methanol as the developing agent (n = 3), ensuring that the obtained RCC_{TLC} effectively minimizes errors related to solubility.

15) It is recommended to include the HPLC analysis of the crude reaction mixture, including UV and gamma traces, at least for several reactions. Due to the complexity of the two-stage one-pot reaction, the chromatographic profile of the radiofluorination mixture is expected to be intricate. This aspect is crucial in real-world PET radiotracer production because it directly affects the chemical purity of radiopharmaceuticals meant for human use. The manuscript does not clearly specify this, and it is challenging to discern from the provided UV trace, which only includes the co-injection of a reference standard in the HPLC chromatogram. The cleanliness of this reaction is crucial for determining the purification effort needed in the use of the short half-life (approximately 110 minutes) radioisotope.

Re: Thanks for the comment. All original preparative HPLC traces for the crude ^{18}F-labeled products have been included in the revised Supplementary Materials (**Figure S2**−**S28**). Each trace clearly identifies the corresponding precursors and standards, as illustrated in **Figure S13**.

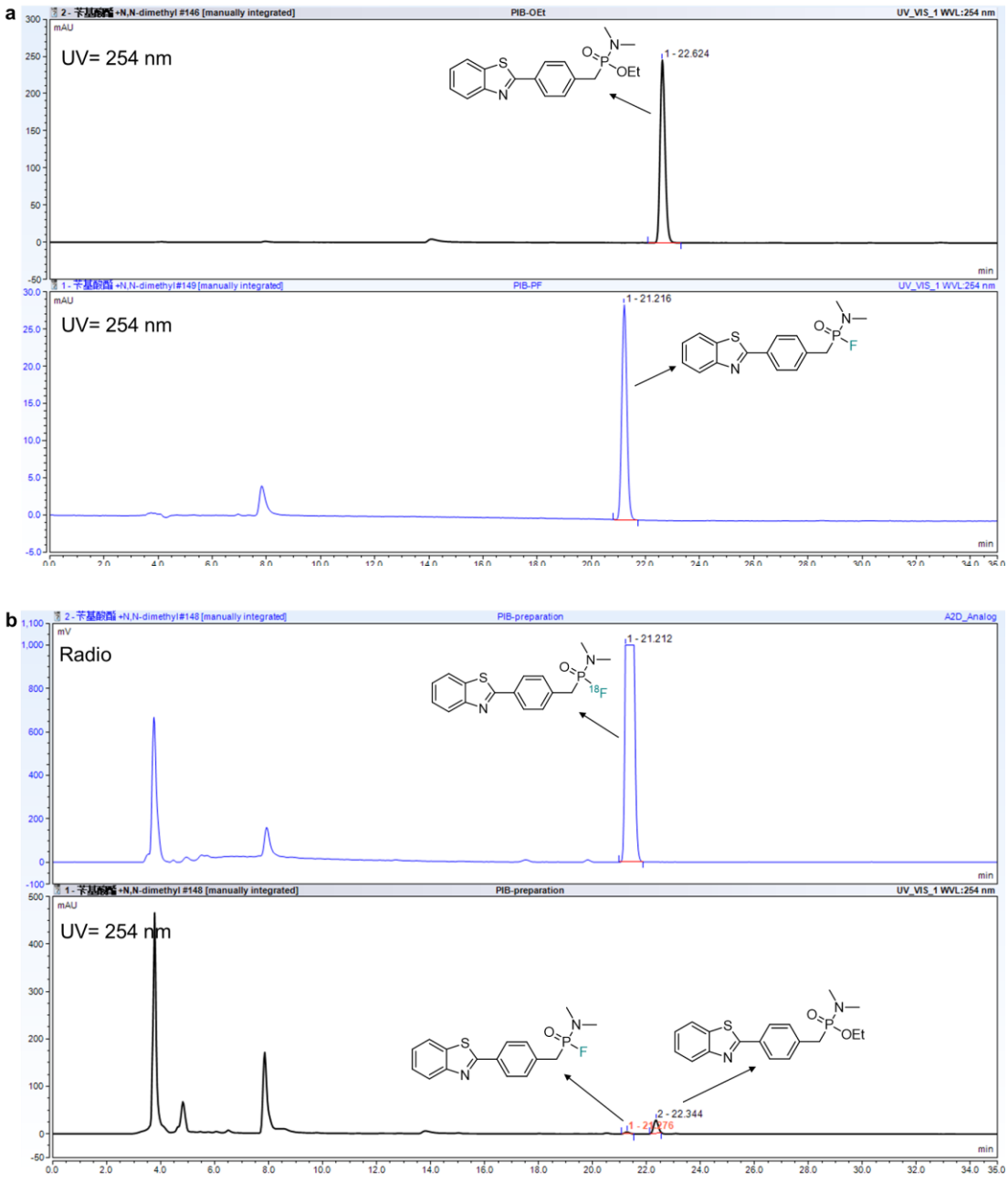


Figure S13. **a**. HPLC traces of precursor and reference compounds; **b.** HPLC traces of crude ^{18}F-labeled products. 1# HPLC conditions; SEP Basic-C18 semi-preparative column (120A 5μm 10 × 250mm); isocratic elution, 0−10 min, from 40% of CH_3CN and 60% of water containing 0.1%TFA to 50% of CH_3CN and 50% of water containing 0.1%TFA; 10−15 min, from 50% of CH_3CN and 50% of water containing 0.1%TFA to 55% of CH_3CN and 45% of water containing 0.1%TFA; 15−18 min, from 55% of CH_3CN and 45% of water containing 0.1%TFA to 60% of CH_3CN and 40% of water containing 0.1%TFA; 18−35 min, 60% of CH_3CN and 40% of water containing 0.1%TFA; flow rate: 3.0 mL/min.

*16) In Supplementary Materials S54 and S55, based on the HPLC and radio-HPLC chromatograms of [^{18}F]**35a** provided, a significant amount of radioactive byproduct is consistently formed (confirmed by three runs and UV trace) near the product peak. Is it possible that sulfide is influencing the production of this undesired radioactive byproduct? Alternatively, can you adjust the HPLC conditions to separate the overlapping sections? This issue is also observed with [^{18}F]**36a** and [^{18}F]AquaF-Flurpridaz.*

Re: Thanks for the comments. We have re-evaluated the HPLC conditions and successfully provided clean radio-HPLC traces for [^{18}F]**44** (representing the original [^{18}F]**36a**) and [^{18}F]**45** (representing the original [^{18}F]AquaF-Flurpiridaz), achieving radiochemical purities greater than 98%. Additionally, the HPLC traces provided for the ^{18}F-labeled products indicate that the presence of sulfur atom did not contribute to the formation of the undesired radioactive byproducts.

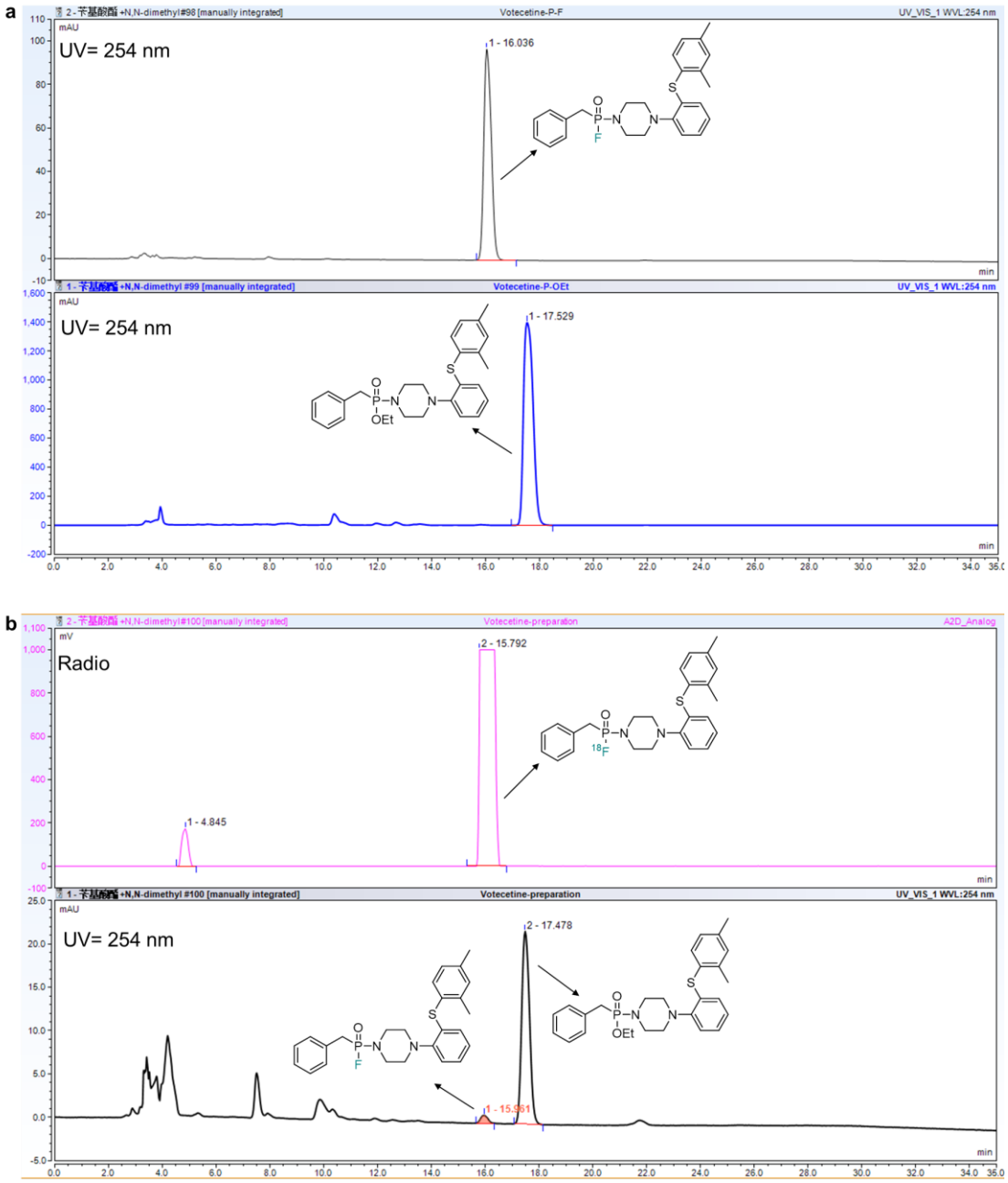


Figure S21. **a**. HPLC traces of precursor and reference compounds; **b.** HPLC traces of crude ^{18}F-labeled products. 1# HPLC conditions; SEP Basic-C18 semi-preparative column (120A 5μm 10 × 250mm); isocratic elution, 0−15 min, from 80% of CH_3CN and 20% of water containing 0.1%TFA to 85% of CH_3CN and 15% of water containing 0.1%TFA; 15−25 min, 85% of CH_3CN and 15% of water containing 0.1%TFA; 25−35 min, from 85% of CH_3CN and 15% of water containing 0.1%TFA to 90% of CH_3CN and 10% of water containing 0.1%TFA; flow rate: 3.0 mL/min.

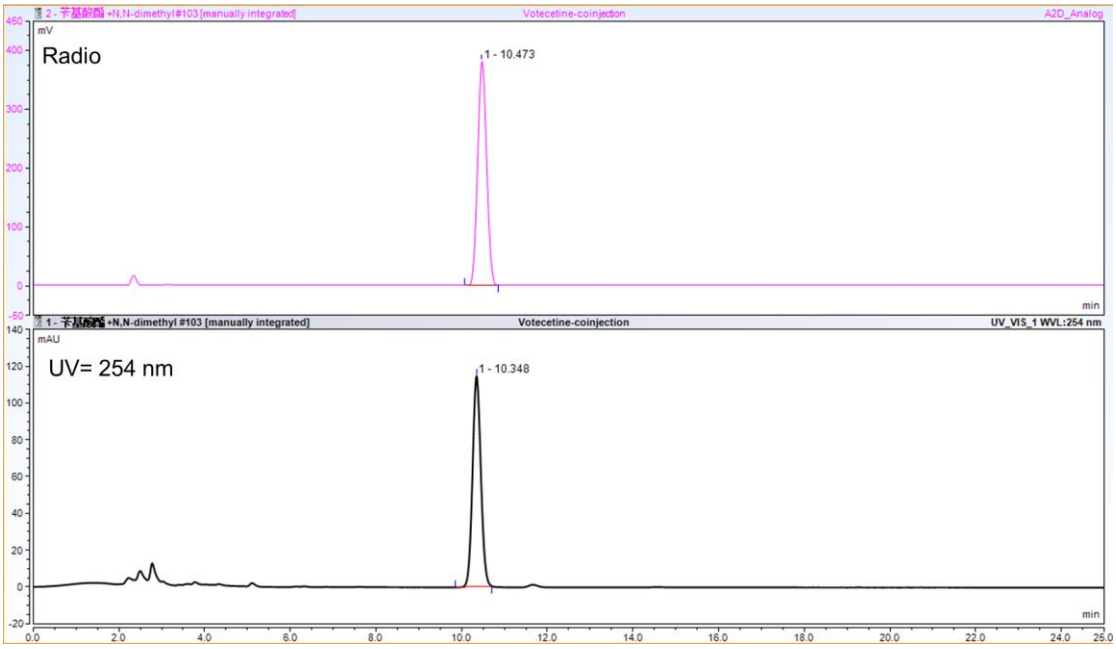


Figure S22. Co-injection for [^{18}F]**44**. 1# HPLC condition: NanoChrom C18 column (5 μm, 4.6 × 250 mm), isocratic elution, water containing 0.1%TFA/CH_3CN = 50/50, and the flow rate was 1 mL/min for 20 min. The RCP was > 98%.

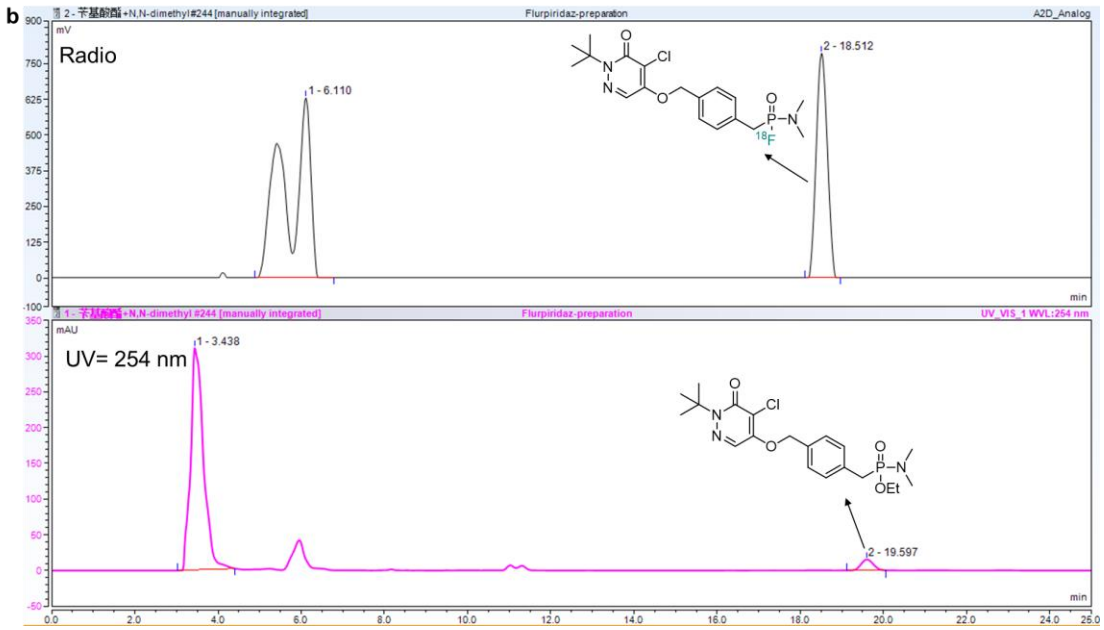


Figure S23. **a**. HPLC traces of precursor and reference compounds; **b.** HPLC traces of crude ^{18}F-labeled products. 1# HPLC conditions; SEP Basic-C18 semi-preparative column (120A 5μm 10 × 250mm); elution: isocratic elution, water containing 0.1%TFA/CH_3CN = 50/50, and the flow rate was 3.0 mL/min for 25 min.

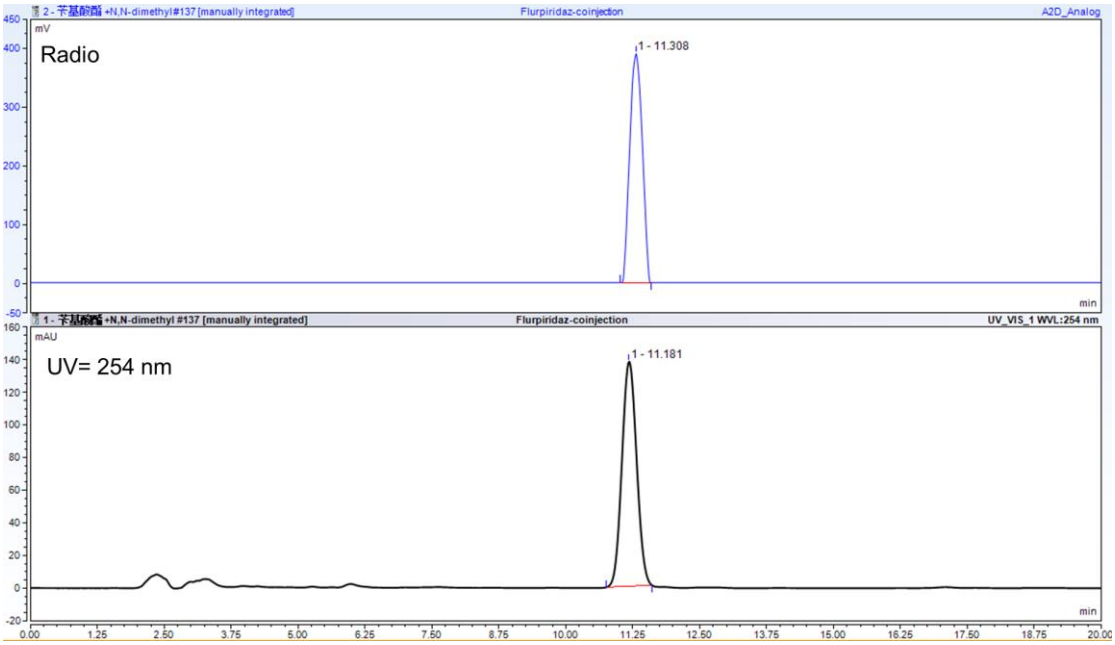


Figure S24. Co-injection for [^{18}F]**45**. 1# HPLC condition: NanoChrom C18 column (5 μm, 4.6 × 250 mm), isocratic elution, H_2O/CH_3CN = 50/50, and the flow rate was 1.0 mL/min for 20 min. The RCP was > 98%.

17) In Supplementary Materials S62, please provide specific details regarding the assay concentration, not just a range. If the protocol was not developed in the laboratory, include a reference to enable replication of the experiment.

Re: Thank you for your comment. The specific details regarding the assay concentration have been included in the revised Supplementary Materials, as well as in the caption in the new **Figure S36** (the IC_{50} curves).

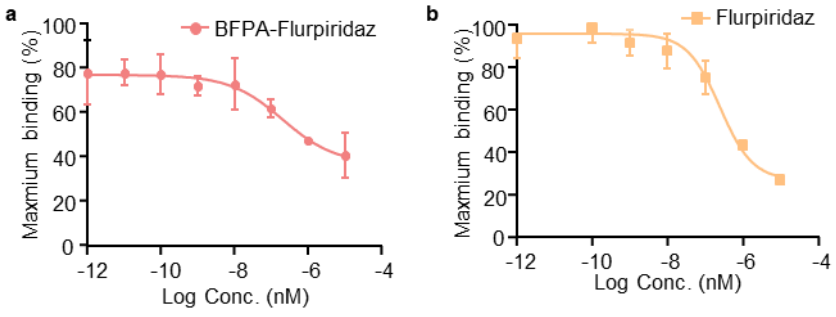


Figure S36. IC_{50} curves of BFPA-Flurpiridaz and Flurpiridaz inhibiting [^{18}F]Flurpiridaz. The IC_{50} value for [^{18}F]BFPA-Flurpiridaz was found to be 148.0 nM, which is significantly lower than the IC_{50} of 248.2 nM for the parent compound Flurpiridaz.

18) In Supplementary Materials S62, it is also advisable to conduct a stability test of the compound using UV trace (refer to Figure 19S, A and B). This is important because under radio-HPLC conditions, only radioactive (gamma) signals can be detected. Therefore, if there is degradation of the non-radioactive chemical entity (as indicated by the UV trace), it does not necessarily imply that [^{18}F]BFPA-E[c(RGDyK)]$_2$ is stable for further biological assessment.

Re: Thanks for the comment. When a new non-radioactive chemical entity is generated from a radioactive compound, it is accompanied by the formation of a new radioactive chemical entity. Therefore, the stability of a radioactive compound can be assessed through the radioHPLC.

19) In Supplementary Materials S59, 60: As previously discussed, employing a two-stage one-pot method on a large scale would be advantageous for the radiopharmaceutical community. To enhance understanding of the radiofluorination process, it is suggested to include a preparative HPLC chromatogram of crude reaction mixture before purification and an analytical HPLC chromatogram to evaluate the integrity of the reaction. This approach is more beneficial than just relying on a correlation curve to calculate molar activity.

Re: Thank you for the suggestion. We have included all radio-preparative HPLC traces for the crude ^{18}F-products in the revised supplemental information (**Figure S2**−**S28**).

20) Throughout the main text and supplementary material, the separate use of ^{19}F- and ^{18}F-notation is unnecessary. Non-radioactive reactions can be simply described as fluorination, fluoro-, etc., while only radioactive reactions or products should be noted with $^{18}F^-$.

Re: Thanks for the comment. This revision has been made in both the revised manuscript and Supplementary Materials to eliminate the need for ^{19}F notation in the context of non-radioactive reactions.

Reviewer #4

In this manuscript, Li and coworkers report the room-temperature-driven nucleophilic fluorination to construct fluorophosphines via active phosphine intermediates. This method enables the late-stage (radio)fluorination of broad dialkyl and monoalkyl phosphonates. The mechanism of this late-stage fluorination was fully explored by experimental and computational studies. The substrate scope is wide, and the fluorination of many bioactive molecules can also be obtained. Furthermore, radiofluorination of medically significant ^{18}F-tracers and synthons are completed, and corresponding PET experiments were conducted. Because of the importance of the late-stage (Radio)fluorination of alkyl phosphonates, I believe that the current investigation is truly useful and of board interests of the chemical society. Nevertheless, several issues are required to be addressed before acceptance.

Re: Thank you for the positive comments. Corresponding responses to each comment have been provided below.

1) In the introduction, the authors should give more reasons why chosen N-heteroaromatic bases (line 82-83).

Re: Thanks for the comment. The following explanation has been added to the introduction of the revised manuscript: "*N*-heteroaromatic bases may serve as active LG through an Arbuzov-like pathway, which are commonly used in Tf_2O-mediated electrophilic activation and stabilize reactive intermediates[40], allowing for broader screening options due to their various electronic substituents." Furthermore, to enhance the systematic research, we also screened non-*N*-heteroaromatic bases (Et_3N, DIPEA, DBU and K_2CO_3) in the revised manuscript, as shown in the new **Figure 2a**.

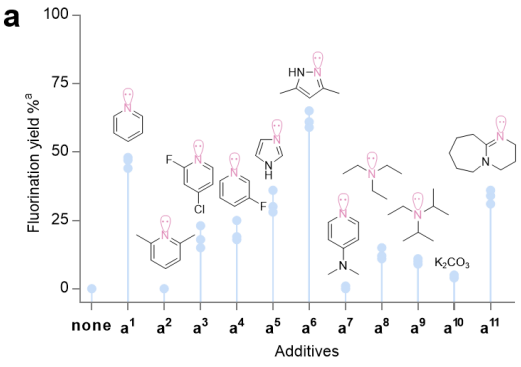


Figure 2. Optimization of conditions for late-stage (radio)fluorination of alkyl phosphonates *via* electrophilic activation. a, Optimization of *N*-heteroaromatic bases, ethyl 4-(((dimethylamino)(ethoxy)phosphoryl)methyl)benzoate (**S2**) as a model compound.

2) In Figure 2a, which N atom will coordinate to P(V) in a5 and a6? I think the lone pair should be drawn on the other N atom.

Re: Thanks for the comment. The coordination in **Figure 2a** has been clarified by drawing the lone pair on the appropriate *N* atom that coordinates to P(V) in $\mathbf{a}^{5}$ and $\mathbf{a}^{6}$.

3) In Figure 2c, Why iPrO group has a higher yield than EtO group? In Figure 3c, OTf-attacking on the methylene of the ester is the rate-determining step. iPr group has a larger steric hindrance than Et group. I speculate that the TS-3 of iPrO group should be higher than that of EtO group.

Re: Thanks for the comment. ^{31}P NMR analysis of the crude reaction products revealed that the lower yields were primarily due to incomplete consumption of starting materials. Fresh Tf_2O was used to repeat all experiments in the new **Figure 2c**, and it was found that steric hindrance had negligible impact on the fluorination yields, with the −*i*PrO substituted yielding 88% and the −EtO substituted yielding 82%. "The results showed that substrates with varying degrees of steric hindrance had negligible impact on fluorination yields. (*Nat Commun* **13**, 4427 (2022). https://doi.org/10.1038/s41467-022-32191-6)" The above description has been incorporated into revised manuscript.

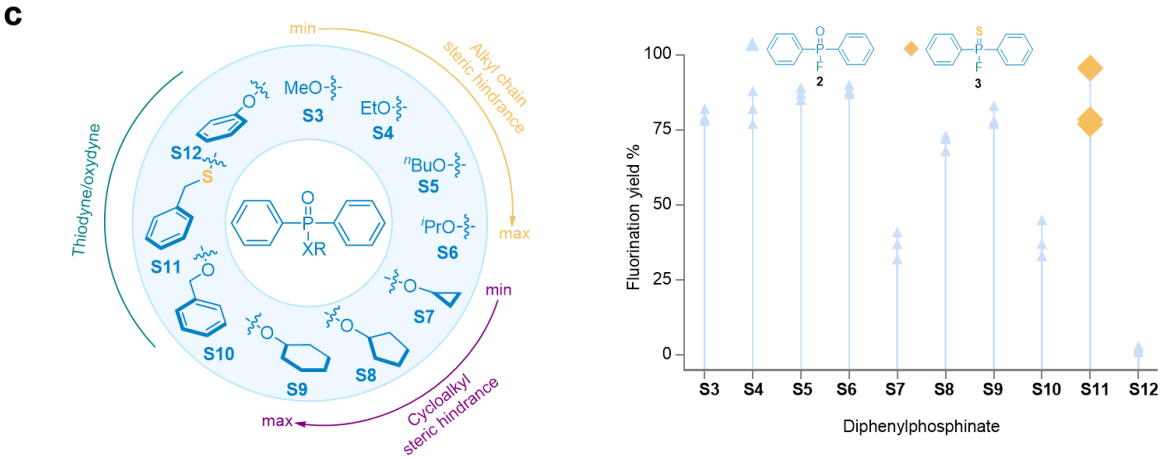


Figure 2c. Study of direct fluorination of different diphenylphosphonates (**S3**−**S12**).

4) Could you please help me explain the content in lines 193 to 196? I don't fully understand now.

Re: Thanks for the comment. The clearer new description intended to be conveyed in the original manuscript on lines 193 to 196 has been incorporated into the revised manuscript. This

new description states: “These results clearly demonstrated that the P−O or P−S single bonds in phosphonates with P−O^{i}Pr or P−SBn groups remained intact during the fluorination of alkyl phosphonates. Instead, cleavage of the C−O or C−S bonds occurred, leading to the separation of isopropyl or benzyl segments and the formation of a new P═X bond, resembling the classical Arbuzov reaction pathway.”

5) Since the scan of the P-N distance indicates that TS-2 may not exist in Figure S30, TS-2 is unnecessary in Figure 3c.

Re: Thanks for the comment. In the revised manuscript, the depiction of **TS-2** has been removed from the new **Figure 4** (formerly **Figure 3c**).

*6) For **26a**, in my opinion, the Ph group in N-methylaniline is an electron-withdrawing group. Therefore, the N-methylaniline group should increase the electrophilicity of P-center rather than decrease it? Perhaps a new explanation is needed for why **26a** fails to generate the desired product.*

Re: Thanks for the suggestion. Experimental findings indicate that the *N*-methylaniline-substituted phosphonate precursor (**S46**, formerly **26a**) fails to react with Tf_2O in the initial step. The original statement has been revised from “possibly due to the significantly reduced electrophilicity of the *P*-center caused by the p-π conjugated system of the electron-rich aniline” to “*N*-methylaniline, as an electron-withdrawing group, can reduce the nucleophilicity of O, which may account for the hindrance encountered by O in attacking Tf_2O”.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The author responded well to my question. I have no further questions.

Re: Thanks for your review.

Reviewer #2 (Remarks to the Author):

The authors have addressed concerns, and the referee suggests accepting the manuscript without further revision.

Re: Thanks for your review.

Reviewer #3 (Remarks to the Author):

The revised manuscript by Zhang et al. properly addresses issues associated with the reviewers ' comments and suggestions. The methodology proposed in this manuscript will enable the efficient generation of diverse alkyl phosphonates through the electrophilic activation of precursor, as well as its relevance to radiochemical applications. I think the manuscript has significantly improved and is worthy of publication in Nature Communications, particularly for its broader audience, including those in the organic and radiopharmaceutical fields.

Re: Thanks for your review.

Reviewer #3 (Remarks to the Author):

The author has thoroughly addressed the reviewers' comments, and I recommend that this manuscript be accepted for publication in Nature Communications. There is only one minor issue: in response to the final comment, it should be clarified that it is the -Ph group in N-methylaniline that is electron-withdrawing, rather than N-methylaniline itself being electron-withdrawing.

Re: Thanks for this comment. We have revised the sentence, changing "*N*-methylaniline, as an electron-withdrawing group" to "since the phenyl (-Ph) group acts as an electron-withdrawing group in *N*-methylaniline".