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Peer Review File

Beyond Traditional Strain-Promoted Azide–Alkyne Cycloadditions by Achieving Orthogonality and Rapid Kinetics with Fluoroalkyl Azides

Corresponding Author: Dr Petr Beier

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)
This manuscript reports the development of fluoroalkyl azides as novel reagents for the strain-promoted azide–alkyne cycloaddition (SPAAC) reaction. The authors demonstrate that fluoroalkyl azides exhibit enhanced reactivity toward electron-rich cyclooctynes such as BCN, while showing significantly reduced reactivity toward electron-deficient dipolarophiles like DIBAC, thereby enabling orthogonal bioconjugation in proteins and living cells. The authors also conducted systematic mechanistic calculations, revealing that electronic complementarity is the key factor governing both reactivity and selectivity. Overall, this work provides a valuable addition to the bioorthogonal chemistry toolkit for SPAAC, enabling dual-target labeling and advancing the design of next-generation molecular probes. However, several aspects of the manuscript require revision.
1.The authors evaluated the stability of azide compound 2a, but only in aqueous solution, which is insufficient. Considering that the probes are designed for use in complex live-cell environments, it is recommended that the authors assess their stability in biologically relevant media, such as cell culture medium, over an extended period of time.
2.Characterizing the reaction orthogonality of the developed fluoroalkyl azide probes is crucial, as this underpins their subsequent applications. However, Figure 4 is currently somewhat confusing. The authors should clearly indicate the positions of different cycloaddition adducts in the LC chromatograms, particularly those corresponding to products with low conversion rates (e.g., in Figure 4C). In addition, the LC peaks should be correlated with their corresponding mass spectra. The authors are also encouraged to cite recent relevant literature on mutually orthogonal bioorthogonal probes for multiplex imaging, such as Angew. Chem. Int. Ed. 2024, e202319853; Angew. Chem. Int. Ed. 2025, e202511705.
3.The overall layout of the figures and schemes could be improved. The manuscript currently contains too many separate figures and tables, which affects readability. Some of them could be combined. For example, Scheme 1 and Table 1 could be merged, as the substituents (R groups) in Table 1 correspond to the structures shown in Scheme 1. Similarly, Scheme 4 and Figures 4 and 5 could potentially be integrated into a single comprehensive figure. In addition, the red underlines beneath “TzMab” and “ConA” in Figure 5 should be removed.

Reviewer #2

(Remarks to the Author)
In their manuscript, Tomčo et al. describe a novel extension of the well-known strain-promoted azide-alkyne cycloaddition (SPAAC) reaction by the introduction fluoroalkyl azides as a reaction partner for strained alkynes. Bioorthogonal strain promoted cycloadditions are an area of great interest to the chemical biology community, as the use of toxic copper in the alternative copper catalyzed azide-alkyne cycloaddition reaction is not fully compatible with experiments in living cells. Overall, I would say this is a well written manuscript on a topic of interest to the wider chemical community. Therefore I suggest publication in communications chemistry should be considered. However, I do have a few points of critique that I think should be addressed:

1. The authors state “[probe 11] reacted less efficiently with DIBAC (Figure 4C)”, but the LC trace of figure 4C to me seems to indicate full conversion to the expected cycloaddition product, based on the retention time shift of the main peak. If however the authors mean that this is only retention time drift of the starting material and the cycloaddition product is the minor peak at

RT ~5.1 min, this should be made more clear in the figure.

2. While I agree with the authors that direct comparison of the signal intensity of the gel bands (figure 5) with different dyes should not be made, I do believe that it is very important for these kinds of experiments to be carried out at equal protein concentrations. The Coomassie stains shown in the SI (Figures SI12-14) indicate quite unequal protein concentrations, with especially ConA-DIBAC showing a lot less intense bands than the other proteins. I think repeating this experiment at equalized protein concentrations would greatly strengthen the results of Figure 5.

3. In scheme 3, the authors show convincingly that the fluoroalkyl azide is stable to amine and thiol nucleophiles under biochemical conditions, which is very good to see. I do wonder how the compatibility with other common biorthogonal reactants, specifically strained alkenes, is. For instance, some cycloaddition reactions between azides and trans-cyclooctene are known (dx.doi.org/10.1021/acs.bioconjchem.7b00665, dx.doi.org/10.1002/anie.201104389) but tetrazine/tco ligation is typically considered orthogonal to azide/alkyne cycloaddition. Could you comment on whether this remains true when using fluoroalkyl azides?

Finally, I believe that the experiment in Figure 6 is key to show the usefulness of the developed SPAAC chemistry to cellular labeling applications. I would strongly suggest enlarging this figure, as the miniscule pictures make the results seem less convincing.

Reviewer #3

(Remarks to the Author)
In this paper, Tomco et al report on the use of fluoroalkyl azides for strain-promoted ‘click’ reactions, and compare their reactivity to more standard alkyl azides. They observe interesting differences in cyclooctyne preference, and I believe that these molecules have very interesting properties that could be of value to the chemical biology community. However, in my opinion there are some major flaws to the research that have been carried out which make the work, in its current form, unsuitable for publication. I would note that many of these flaws could be addressed with relatively simple experiments though, and I believe that underpinning the paper is some very meritorious science. My major comments are:
i) The whole premise of the work is to use these molecules for bioconjugation, i.e. under aqueous conditions. However, all kinetic experiments were performed in solely organic solvent. I realise there are solubility issues here, but it is common in similar work to include at least 10% water, due to the known ability of cycloadditions to be strongly influenced by its presence. For example, they compare reactivity to 4-azidopyridine data previously reported, but that work WAS done in at least partially aqueous solvent. The absence of water in all small molecule work means that it is then difficult to draw correlations to any of the subsequent protein work, potentially manifesting in some of the comments below
ii) The IR data in Figure 2 appears to have been either edited in some way, or converted poorly, since there are large bits of the expected continuous line missing. I am pretty confident it is the latter, but it needs addressing. More generally, this should be an SI rather than main figure
iii) The computational data is not consistent with the experimental data, contrary to what the authors write. Though there is a difference in reactivity predicted for 4, and this has the right relevant position to the reactivity with 2a, the BCN and DIBAC have almost identical Ea for 2a despite an observed 100 fold difference in reactivity. The data cannot therefore be trusted as having strong predictive power.
iv) Competition reactions with lysine and thiols are performed in water, but the pH of these reactions is not clear, yet very important. Either the pH needs measuring, or they need to be performed in buffered conditions to be relevant to the discussion.
v) Cf. point 1, the mixtures of labelled protein obtained in Figure 5 are not in line with predictions. Based on the authors results, with a BCN-modified protein the fluorinated azide should exhibit a drastically increased rate of reaction and therefore labelling should be specific. That the data after seems to indicate that reactivity is protein-environment dependent is not surprising, but also takes away from the premise of the paper that selectivity can be achieved through suitable choice of azide.
In addition, I have the following minor comments that I hope the authors can use to improve the manuscript:
i) Page 1, is it really correct to call BCN an electron rich alkyne? It’s not electron-deficient like the others, but that’s not the same
ii) I found it difficult to understand where the data in Table 1 came from
iii) It wasn’t clear to me why a cyclononyne and decyne were also investigatred, is there precedence for the reactivity of these in SPAAC?
iv) I found it difficult to interpret figure 4, it wasn’t clear what peaks were vs what expected product was

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript has been improved after revision, and I recommend it for publication.

Reviewer #2

(Remarks to the Author)
I am satisfied with the authors' responses and recommend publication of the manuscript.

Reviewer #3

(Remarks to the Author)
I am satisfied that my comments have been addressed and for the paper to be accepted for publication

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript reports the development of fluoroalkyl azides as novel reagents for the strain-promoted azide–alkyne cycloaddition (SPAAC) reaction. The authors demonstrate that fluoroalkyl azides exhibit enhanced reactivity toward electron-rich cyclooctynes such as BCN, while showing significantly reduced reactivity toward electron-deficient dipolarophiles like DIBAC, thereby enabling orthogonal bioconjugation in proteins and living cells. The authors also conducted systematic mechanistic calculations, revealing that electronic complementarity is the key factor governing both reactivity and selectivity. Overall, this work provides a valuable addition to the bioorthogonal chemistry toolkit for SPAAC, enabling dual-target labeling and advancing the design of next-generation molecular probes. However, several aspects of the manuscript require revision.
1.The authors evaluated the stability of azide compound **2a**, but only in aqueous solution, which is insufficient. Considering that the probes are designed for use in complex live-cell environments, it is recommended that the authors assess their stability in biologically relevant media, such as cell culture medium, over an extended period of time.

Author: We performed additional stability experiment in biologically relevant media. Azide **2a** was treated with 10% FBS (Fetal Bovine Serum) in DMEM (Dulbecco's Modified Eagle Medium). After 3 h at room temperature azide **2a** was found to be stable by 19F NMR (Scheme 4A). Thus, azide **2a** is stable not only in aqueous buffer but also in a complex medium.

2.Characterizing the reaction orthogonality of the developed fluoroalkyl azide probes is crucial, as this underpins their subsequent applications. However, Figure 4 is currently somewhat confusing. The authors should clearly indicate the positions of different cycloaddition adducts in the LC chromatograms, particularly those corresponding to products with low conversion rates (e.g., in Figure 4C). In addition, the LC peaks should be correlated with their corresponding mass spectra. The authors are also encouraged to cite recent relevant literature on mutually orthogonal bioorthogonal probes for multiplex imaging, such as Angew. Chem. Int. Ed. 2024, e202319853; Angew. Chem. Int. Ed. 2025, e202511705.

Author: We modified Figure 4 (Figure 2 after revision) to make things clear. In the SI we added MS spectra for the respective HPLC signals. In the manuscript (Figure 2A), we showed only the HPLC traces of the reaction mixture (azide **11** with BCN and DIBAC) and indicated the identity of all signals.

3.The overall layout of the figures and schemes could be improved. The manuscript currently contains too many separate figures and tables, which affects readability. Some of them could be combined. For example, Scheme 1 and Table 1 could be merged, as the substituents (R groups) in Table 1 correspond to the structures shown in Scheme 1. Similarly, Scheme 4 and Figures 4 and 5 could potentially be integrated into a single comprehensive figure. In addition, the red underlines beneath "TzMab" and "ConA" in Figure 5 should be removed.

Author: We combined Scheme 1 and Table 1 into new Scheme 1. We also simplified Table 2 (Table 1 after revision), moved original Figure 2 (IR spectra) to the SI, and improved the visibility of Figure 6 (Figure 4 after revision).

Reviewer #2 (Remarks to the Author):

In their manuscript, Tomčo et al. describe a novel extension of the well-known strain-promoted azide-alkyne cycloaddition (SPAAC) reaction by the introduction fluoroalkyl azides as a reaction partner for strained alkynes. Bioorthogonal strain promoted cycloadditions are an area of great interest to the

chemical biology community, as the use of toxic copper in the alternative copper catalyzed azide-alkyne cycloaddition reaction is not fully compatible with experiments in living cells. Overall, I would say this is a well written manuscript on a topic of interest to the wider chemical community. Therefore I suggest publication in communications chemistry should be considered. However, I do have a few points of critique that I think should be addressed:

1. The authors state "[probe 11] reacted less efficiently with DIBAC (Figure 4C)", but the LC trace of figure 4C to me seems to indicate full conversion to the expected cycloaddition product, based on the retention time shift of the main peak. If however the authors mean that this is only retention time drift of the starting material and the cycloaddition product is the minor peak at RT ~5.1 min, this should be made more clear in the figure.

Author: Figure 4 (Figure 2A after revision) was modified to clearly indicate the position of starting coumarin-azide **11** and SPAAC product **16**. The product of SPAAC reaction of azide **11** with DIBAC did not form. Control HPLC traces and MS spectra are now in the SI.

2. While I agree with the authors that direct comparison of the signal intensity of the gel bands (figure 5) with different dyes should not be made, I do believe that it is very important for these kinds of experiments to be carried out at equal protein concentrations. The Coomassie stains shown in the SI (Figures SI12-14) indicate quite unequal protein concentrations, with especially ConA-DIBAC showing a lot less intense bands than the other proteins. I think repeating this experiment at equalized protein concentrations would greatly strengthen the results of Figure 5.

Author: Our initial aim was to modify both proteins (trastuzumab and Concanavalin A) in the same way so that we could use the same protein concentration in the experiment. However, we repeatedly observed differences in the amount of attached BCN and DIBAC dipolarophile, even though we used the same amount of NHS active esters and the same conditions. It seems that the coupling reaction is protein-specific, and the degree of labeling (measured by MS) varied between the two proteins. It also depends on the specific combination (Trastuzumab + DIBAC-NHS, Trastuzumab + BCN-NHS, ConA + DIBAC-NHS, ConA + BCN-NHS all resulted in different DOL). Therefore, to ensure **equal concentrations of dipolarophiles attached to each protein**, we adjusted protein concentrations. This explains the different band intensities observed in the gels. In this type of experiment, matching the dipolarophile concentration is crucial. We know this isn't ideal; however, preparing site-specific, uniformly modified proteins is challenging and currently beyond the scope of this manuscript. We also acknowledge a limitation: the click reactivity of the two azides can vary within the protein context. We mention this in the manuscript to inform readers and those interested in applying our method for simultaneous protein labeling. On the other hand, we observed excellent selectivity in cellular experiments, which we believe better demonstrates the practical utility of our approach.

3. In scheme 3, the authors show convincingly that the fluoroalkyl azide is stable to amine and thiol nucleophiles under biochemical conditions, which is very good to see. I do wonder how the compatibility with other common biorthogonal reactants, specifically strained alkenes, is. For instance, some cycloaddition reactions between azides and trans-cyclooctene are known (dx.doi.org/10.1021/acs.bioconjchem.7b00665, dx.doi.org/10.1002/anie.201104389) but tetrazine/tco ligation is typically considered orthogonal to azide/alkyne cycloaddition. Could you comment on whether this remains true when using fluoroalkyl azides?

Author: We performed a control experiment to assign the compatibility of our SPAAC reaction with the ligation using trans-cyclooctene (TCO) with a tetrazine (Figure 2B). A mixture of tetrazine, azide **11** and TCO produced only the adduct **19** (reactivity of TCO with tetrazine). No product of click reaction of **11** with TCO was observed. The relevant reference: dx.doi.org/10.1021/acs.bioconjchem.7b00665 was added (new ref. 34).

Finally, I believe that the experiment in Figure 6 is key to show the usefulness of the developed SPAAC chemistry to cellular labeling applications. I would strongly suggest enlarging this figure, as the miniscule pictures make the results seem less convincing.

Author: We modified Figure 6 (Figure 4 after revision) to be larger and better visible.

Reviewer #3 (Remarks to the Author):

In this paper, Tomco et al report on the use of fluoroalkyl azides for strain-promoted 'click' reactions, and compare their reactivity to more standard alkyl azides. They observe interesting differences in cyclooctyne preference, and I believe that these molecules have very interesting properties that could be of value to the chemical biology community. However, in my opinion there are some major flaws to the research that have been carried out which make the work, in its current form, unsuitable for publication. I would note that many of these flaws could be addressed with relatively simple experiments though, and I believe that underpinning the paper is some very meritorious science. My major comments are:
i) The whole premise of the work is to use these molecules for bioconjugation, i.e. under aqueous conditions. However, all kinetic experiments were performed in solely organic solvent. I realise there are solubility issues here, but it is common in similar work to include at least 10% water, due to the known ability of cycloadditions to be strongly influenced by its presence. For example, they compare reactivity to 4-azidopyridine data previously reported, but that work WAS done in at least partially aqueous solvent. The absence of water in all small molecule work means that it is then difficult to draw correlations to any of the subsequent protein work, potentially manifesting in some of the comments below

Author: The kinetic measurements were in fact performed in a THF/water mixture (9:1) as indicated in the text. The information about the solvent system used was added to Figure 1 caption to make the used solvent system more evident.

ii) The IR data in Figure 2 appears to have been either edited in some way, or converted poorly, since there are large bits of the expected continuous line missing. I am pretty confident it is the latter, but it needs addressing. More generally, this should be an SI rather than main figure

Author: The IR spectra of studied compounds were measured in chloroform. The bands corresponding to chloroform were subtracted from the spectra, resulting in the discontinuous lines. It is common in IR spectroscopy to substract solvent from the spectrum. The azide stretching vibration region (cca 2100 cm^{-1}) is silent for other functional groups present. The IR spectra were converted to a picture of a better resolution, removed from the main text and added to SI.

iii) The computational data is not consistent with the experimental data, contrary to what the authors write. Though there is a difference in reactivity predicted for 4, and this has the right relevant position to the reactivity with 2a, the BCN and DIBAC have almost identical Ea for 2a despite an observed 100 fold difference in reactivity. The data cannot therefore be trusted as having strong predictive power.

Author: We agree that our quantum-chemical data on isolated molecules do not have a strong predictive power for the absolute rate constants. Our intention was more modest: (i) to confirm that all four SPAAC reactions have accessible activation barriers compatible with the experimental rates and (ii) to probe whether standard frontier-orbital descriptors (HOMO/LUMO gaps and "normal vs. inverse electron demand") can rationalize the observed selectivity, i.e. whether the differences can be rationalized in terms of electronic interactions. The answer was positive for the case (i) and negative for the case (ii). Based on our extensive calculations for SPAAC reactions, we believe that the previously published guidelines based on single molecules are not sufficiently robust.

We have revised the manuscript to make this limitation explicit. In the Results section we now clarify that the calculations are used only to support qualitative trends and mechanistic insights, not to reproduce absolute rate constants, and we explicitly state that the numerical agreement with experiment is at best semi-quantitative (see revised text on pages 3 and 4 as well as a changed text in the SI).

iv) Competition reactions with lysine and thiols are performed in water, but the pH of these reactions is not clear, yet very important. Either the pH needs measuring, or they need to be performed in buffered conditions to be relevant to the discussion.

Author: We measured the apparent pH of the reaction mixtures with the two additives (10 equiv. of L-lysine or glutathione). pH of the reaction mixture with L-lysine was 9.6. pH of the reaction mixture with glutathione was 3.35. These solutions are buffered by the used excess of the additive and pH did not change during the reaction. Product **3c** was the only product observed by ^{19}F NMR.

v) Cf. point 1, the mixtures of labelled protein obtained in Figure 5 are not in line with predictions. Based on the authors results, with a BCN-modified protein the fluorinated azide should exhibit a drastically increased rate of reaction and therefore labelling should be specific. That the data after seems to indicate that reactivity is protein-environment dependent is not surprising, but also takes away from the premise of the paper that selectivity can be achieved through suitable choice of azide.

Author: Indeed, the reactivity is influenced by the specific protein-dipolarophile combination. We comment on this observation in the main text: "These results demonstrate that both the dipolarophile and the protein context influence the selectivity of the SPAAC reaction" to inform readers and those interested in applying our method for simultaneous protein labeling. This known limitation of protein labeling thus affects selectivity, but the trend toward preferential azide reactivity remains. In summary, all the data from our protein labeling experiments show that **this preferential reactivity is maintained through the appropriate choice of azide, although the extent of this reactivity varies depending on the protein environment**. As we also addressed in response to the second reviewer's comment, we observed excellent selectivity in cellular experiments, which we believe better illustrates the practical utility of our approach.

In addition, I have the following minor comments that I hope the authors can use to improve the manuscript:
i) Page 1, is it really correct to call BCN an electron rich alkyne? It's not electron-deficient like the others, but that's not the same

Author: BCN is relative to DIBAC more electron rich. Data from ref. 19 (DOI: 10.1038/ncomms6378) also suggest it. We changed the main text, so BCN is called "more electron-rich" than DIBAC.

ii) I found it difficult to understand where the data in Table 1 came from

Author: The data in Table 1 (Scheme 1B after revision) come from references 10-18. See the text and Scheme 1 caption.

iii) It wasn't clear to me why a cyclononyne and decyne were also investigatred, is there precedence for the reactivity of these in SPAAC?

Author: We prepared cyclononyne and cyclodecine derivatives to compare them to cyclooctynes. The observation is that they also click with azides, but the reactivity is much lower than for cyclooctynes. Sulfur-containing cyclononyne was not investigated in SPAAC. The cyclodecyne easily accessible from 4,4'-biphenol was previously shown to be reactive in SPAAC with non-fluorinated azides, see refs:

https://doi.org/10.1016/j.chempr.2017.07.011 https://doi.org/10.1039/C7OB00991G https://doi.org/10.1039/D3OB01712E

iv) I found it difficult to interpret figure 4, it wasn't clear what peaks were vs what expected product was

Author: Figure 4 (Figure 2A after revision) was modified to clearly indicate the position starting coumarin-based azide **11** and the only product **16** in reaction of **11** with BCN and DIBAC. Other traces were removed and are now in SI including the MS spectra.