ETH
Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

Laboratory for Physical Chemistry

ETH Zürich
HCI F 225
Wolfgang-Pauli-Str. 10
8093 Zurich
Switzerland

Roland Riek, PhD
Professor for Physical Chemistry
+41 44 632 6 139
+41 44 633 14 48
roland.riek@phys.chem.ethz.ch

Prof. Dr. Jörg Matysik
Editor

Magnetic Resonance

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Re: Submission of the second revised version of the manuscript entitled "Exploration of the close chemical space of tryptophan and tyrosine reveals importance of hydrophobicity in CW-photo-CIDNP performances" by Torres et al.

Dear Jörg

Please find therein the second revised version of the manuscript entitled "Exploration of the close chemical space of tryptophan and tyrosine reveals importance of hydrophobicity in CW-photo-CIDNP performances" by Torres et al. for publication in your esteemed journal within the Festschrift to the birthday of Rob Kaptein. The revised manuscript has been evaluated by two additional reviewers (in addition to the two initial reviews). While reviewer 3 had only minor corrections reviewer 4 placed the attention to potential different pKa effects of the various compounds with the potential to interfere with the findings on the hydrophobicity SNE correlation found at pH 7. While this suggestion is well funded and also observed in a pH titration study performed (Figure S2) the correlation between SNE and hydrophobicity is still observed well above the pH sensitive region of the SNE (Figure S3). Please find more detailed answers to the questions of the reviewer below.

I sincerely hope that the second revision is now well suited for publication.

Sincerely,

Point by point response to the requests and suggestions of the reviewers

Reviewer #3
Reviewer #3: The term CV should be used in this common notation as continuous wave not as constant wave as on page 3, line 68, for example.

Answer: We would like to thank the reviewer for careful reading. Corrections are done as suggested.

Reviewer #4
Reviewer: The manuscript describes the exploration of chemical space of two aromatic amino acids in CW-photo-CIDNP. Whereas TR-photo-CIDNP would be mechanistically more informative, the approach using CW-photo-CIDNP has clear sensitivity advantages. With their approach the authors could demonstrate the existence of several compounds showing strong photo-CIDNP effects.

In cyclic reactions, as studied here, cancellation of polarization of recombination and escape products (chemically the same in a cyclic reaction) will occur. The observed CIDNP depends critically on the various kinetic rates and nuclear spin relaxation times. Thus not only for understanding the mechanism, but also for optimal CW-CIDNP intensity and identifying new compounds exploration of experimental conditions can play a significant role. The authors could comment on this.

Answer: We agree with this statement of the reviewer that the exploration of experimental conditions are important. In this context, we extended in the revised version the experimental conditions to a pH range plus/minus 2 units around the physiological pH of 7 (Figure S2 and S3). Our interest in exploring CIDNP in drug related research constraints the conditions however significantly (including in addition to a pH around 7-8 also low compound concentration).

Reviewer: Recommendable overviews for this study would be Hore and Broadhurst (1993), Photo-CIDNP of Biopolymers, Prog. NMR Spectrosc. 25, 345-402 and Kuhn (2013), Photo-CIDNP NMR Spectroscopy of Amino Acids and Proteins. In: Kuhn (eds) Hyperpolarization Methods in NMR Spectroscopy. Topics in Current Chemistry Vol 338, Springer Berlin Heidelberg, pp. 229-300, https://doi.org/10.1007/128_2013_427
A related study showing examples CW-photo-CIDNP of tyrosine and tryptophan derivatives would be Stob and Kaptein (1989), Photo-CIDNP of the amino-acids, Photochem. Photobiol. 49(5):565-577, https://doi.org/10.1111/j.1751-1097.1989.tb08425.x. Also the review by Hore and Broadhurst summarizes several dyes and compounds for photo-CIDNP.

Answer: We would like to thank the reviewer for the references, which are now incorporated into the manuscript.

Reviewer: Scheme II and scheme I in the paper by Stob and Kaptein may provide a simple framework to interpret the results in this manuscript as well. That paper also shows a pH and concentration dependency of the CW CIDNP intensity. Fig. 15 in that paper may be a warning, since small [Trp] concentrations can have a steep CIDNP intensity effect, idem Fig. 14 showing strong pH dependency at pH 6. Also the review by Hore and Broadhurst shows such pH dependency for Tyr and Trp (Fig.6, and explained on p.363). The referred paper by Okuno and Cavagnero (2016, J Phys Chem B) also shows possibilities for simulating CW-photo-CIDNP and parameters for that.

The pH dependency of CW-photo-CIDNP intensities could also deviate substantially from the pKa's of compounds. Thus characterizing CW-photo-CIDNP at a few concentrations and pH values may be recommendable for this type of screening. Whether concentration and pH could indeed play a role, the authors could partially check this already using the equations given by Stob and Kaptein.

Answer: To elaborate on the pH effect of the CW-photo-CIDNP we extended the TRP analog studies over a pH range between 5-9 with both dies AT12 and fluorescein (Figure S2) knowing however that our interest is the physiological pH range 7-8. As expected by the reviewer and now discussed in the manuscript a strong pH effect is observed. In this study, we had however to change the oxygen scavenger system as the enzyme cocktail is limited to physiological conditions yielding an overall significant drop of the signal enhancement. As stated above by the reviewer, CW-photo-CIDNP depends on the conditions -).

Reviewer: Though not really observable when perfect 90° detection pulses were used (better detected with 45° pulses), the spectral difference between Tyr and TyrA may also point to a difference in a multiplet effect, for which there is another Kaptein' CIDNP rule, and which would be independend of a diference in g-values in the radical pair and may therefore still be present in case of weak net effects.

Answer: Following the hypothesis of the reviewer we measured the spectra with 45 degree pulse (Figure S4 and Figure S5 below) not finding indication that would support this hypothesis. This experiments were not taken up in the manuscript.

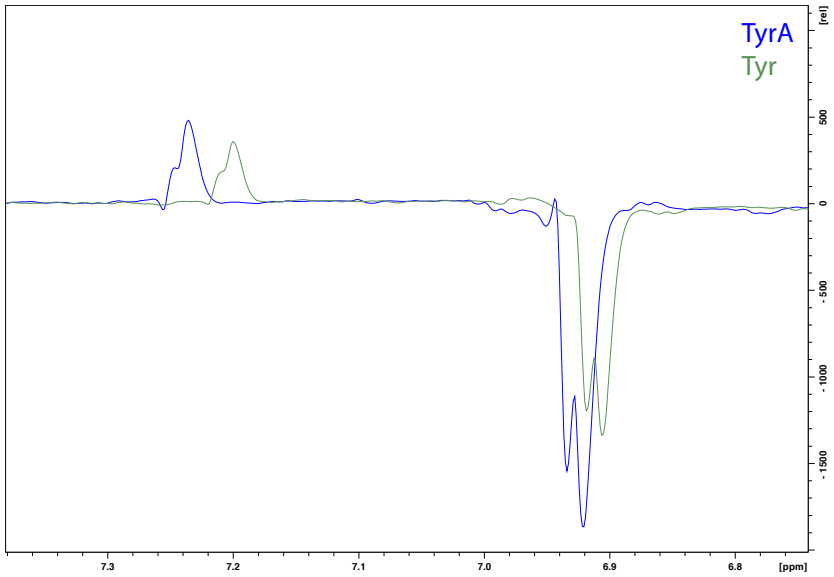

Figure S1: Photo-CIDNP spectra of tyrosine (Tyr) and tyramine (TyrA), with 45° detection pulses. Zoom on the aromatics. The samples were concentrated at 100 μM of Tyr/TyrA and 25 μM of AT12.

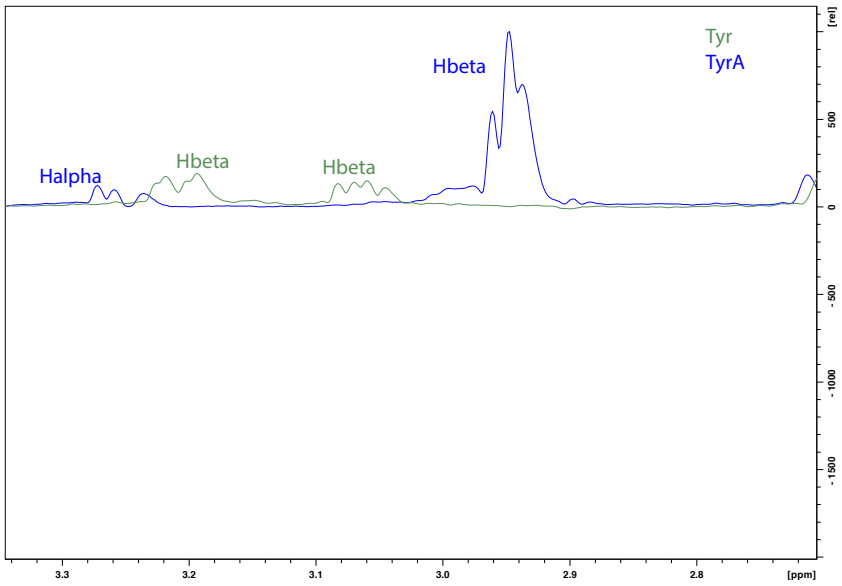

Figure S2: Photo-CIDNP spectra of tyrosine (Tyr) and tyramine (TyrA), with 45° detection pulses. Zoom on the aliphatics. The samples were concentrated at 100 μM of Tyr/TyrA and 25 μM of AT12.

Reviewer: In Table 1, I would use / add the same short names and order as in Fig. 1 (then short names are also defined, and not partially as now in Fig. 2). Please check SNE's in Fig .1 and Table 1: e.g. AT12, SNE for TyrA is -60 (Table 1) and -63 (Fig. 1), whereas with the other molecules numbers appear to be the same. Conditions for the experiment are also the same.

Answer: We would like to thank the reviewer for his careful reading. We followed his suggestions.

Reviewer: The authors may also consider at several instances pKa changes also source for the CIDNP intensity differences:
- Could pKa differences also be explanation for CIDNP intensity changes between HOPI / dH-Trp, IPA / IAA, etc?

Answer: Indeed, based on the new measurements at various pHs, pKa differences are a partial explanation to the differences in the photo-CIDNP intensities, especially in the case of the IAA and IPA. However, the log(P) contribution tendency was still present at different pH (Figure S3, Figure 4).

Reviewer: l. 168: interestingly, such pKa changes had been discussed by Stob and Kaptein (1989) for Trp and N-acetyl-Trp. Could such pKa play a role here as well?

Answer: The differences are between TRP and indole/N-methyl-TRP. The N-acetyl and TRP show quite a small difference especially if one considers the accuracy of the measurement exhibited in the figure 5 of the Stob and Kaptein paper.

Reviewer: l.184/5: are these the estimated pKa's for fluorescein that of its radical? Idem for the other (notably 'tryptophan') pKa's on p.8.

Answer: The pKa here mentioned are the pKa of the ground state molecules and now stated as such in the manuscript. We considered these ones in this part as we discuss the quenching rates which are before the formation of the radical pair.

Reviewer: l. 205: please note that the precise pKa value and thus changes therein could also play a significant role on the CW-CIDNP effects studied here as well. That would much more subtile than overall charge, and could also be explanation for failure of just using charge alone.

Answer: This suggestion was put in practice by measuring the pH titration, and the log(P) dependencies at different pH (Figure S2 and S3). Please see above answers for more details. It was interesting to see that the carboxylic containing analogues are actually the best polarized.

Reviewer: l. 210: the rates of sidechain dynamics may be much slower than the various rates in CIDNP (reactions, ET, protonation). Dynamics would then rather be: presenting more or less active or conformations, or conformations with different pKa's.

Answer: This is an interesting point. The pKa of interest here is the indolyl pKa. The measurement at high pH (i.e. pH 9) where the protonation of the indolyl was everywhere the same should give insights into this hypothesis. As found in Figure S3 the effect under investigation was still observed at high pH challenging the hypothesis. The sidechains dynamics may be potentially involved in the rearrangement dynamics of the water shell around the molecule. This could have an effect on the radical pair formation. This is however pure hypothesis but would fit with the Marcus theory.

Reviewer: l. 222/ Fig.4: the observed correlation of CIDNP SNE and hydrophobicity is interesting. But possibly pKa effects present also a good explanation for observed effetcts. The authors could discuss this as alternative to hydrophobicity.

Answer: As mentioned above already several time this important suggestion was put into practice by measuring the pH titration (Figure S2), and the log(P) dependencies at different pH (Figure S3) and by a new paragraph reading:

"In addition, the pH dependency of the photo-CIDNP SNE where recorded for the different tryptophan analogues, in a pH range between 5 and 9 within 2 units proximity of physiological conditions. While the pKa of the indole is typically around 16, the pKa of the indolyl group has been observed to be rather in the range 7-8 [Kaptein Stob ref]. The latter pKa is known to have a significant influence on the photo-CIDNP hyperpolarization performances. The pH-dependent photo-CIDNP performances of the five tryptophan analogues (TRP, TRA, PEI, IPA, and IAA) for both dyes show overall similar behaviors but group in presence of fluorescence into TRP and TRA with a maximum already at pH 7 and IPA, PEI, and IAA with a maximum at pH 8 while the relative enhancement of IAA at pH 7 is significantly lower when compared with the other compounds (Figure S2B). In presence of AT12 the differences are less manifested with a maximum enhancement reached for pH > 9 (Figure S2B). Similar results have been observed by Stob and Kaptein, for tryptophan and N-acetyl tryptophan. Importantly, the log(P) dependencies were still observed at the optimal pH hereabove mentioned as the plots in Figure S3 show similar correlation as observed in Figure 4, at the difference that the IPA and IAA show the best enhancements for the photo-CIDNP spectra monitored with AT12 and fluorescein. While the common

feature between these two compounds is the carboxylic acid ending the sidechain, the ionic interaction with the dye cannot explain simply these better performances, as the dye charges are significantly different (Figure 1). Moreover, the significantly better SNE for PEI as compared to TRA and TRP is in favor of a positive influence of the hydrophobicity. The extension of this hypothesis to a broader set of molecules may be beneficial to confirm the positive impact of the carboxylic acid in comparison to other negatively charged molecules, and the impact of hydrophobicity on the photo-CIDNP performances.
“

Reviewer: ‘Absolute’ may still be hard, but would relative pKa predictions using DFT calculations of classes of ‘tyrosine’ and ‘tryptophan’ compounds be useful for interpreting the CIDNP intensities, as an additional approach to charge/log(P) or TR-photo-CIDNP?

Answer: We looked into potential calculations of the pKa with DFT and found that on top of its time-intensive computing nature the accuracy is not that great and that experiments can be done much faster. Since from an experimental group point of view the experiment is the gold standard, we decided to do the pH titration as shown in Figure S2.