Report of the Max-Buchner research project

„Glucosinolate (GSL) derived, bio responsive labelling probes for fluorescence imaging of different enzyme activities“ *(MBFSt-Kennziffer: 3743)*

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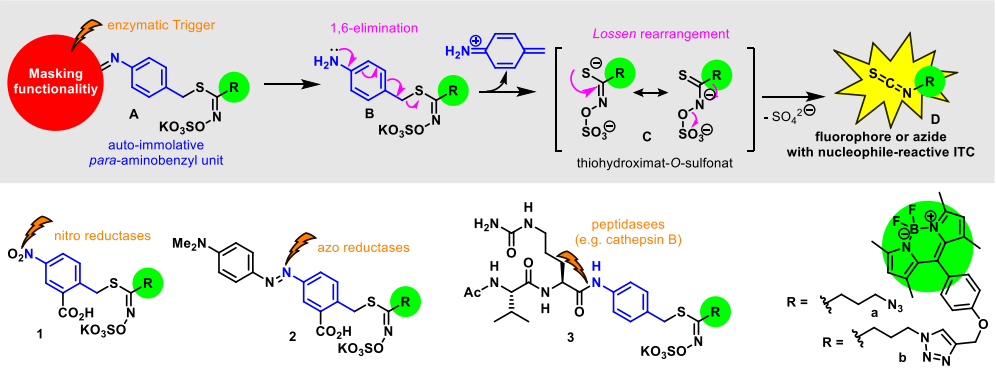
1. Task and aims

Based on our recent work on artificial fluorescent GSLs,[1] we envisaged to adapt the formation of thiohydroximate-*O*-sulfonates to different enzymatic triggers. Therefore, we wanted to synthetically incorporate auto-immolative *para*-amino benzyl units, whose immolation reactivity is masked by a specific, enzymatically labile functional moiety (e.g. compounds **1-3a/b**, Scheme 1).

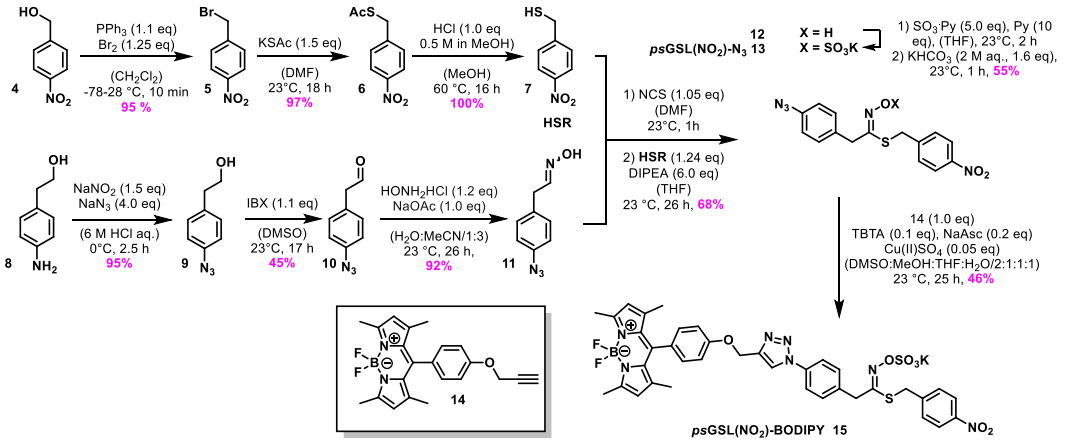


Scheme 1: Novel artificial, fluorescent and azide-containing GSL-derived, bio responsive probes for imaging of different enzyme activities

In the presence of the corresponding enzyme, the *para*-amino benzyl moieties are supposed to be unmasked and release thiohydroximate-*O*-sulfonates through a 1,6-elimination which should lead to the spontaneous formation of ITCs via *Lossen* rearrangement analogous to the natural GSL breakdown. The fluorescent or azide-containing ITCs generated would in a biological context, lead to an irreversible labeling of surrounding cells and tissue in a biological context or could be utilized to modify material surfaces in an enzymatically controlled manner. Thereby, I envisaged to develop an universal molecular tool for fluorescence labeling, which can be easily adapted to different fluorophores and different target enzymes, such as nitro reductases, azo reductases, or peptidases (e.g. cathepsin B[2,3]).

2. Results

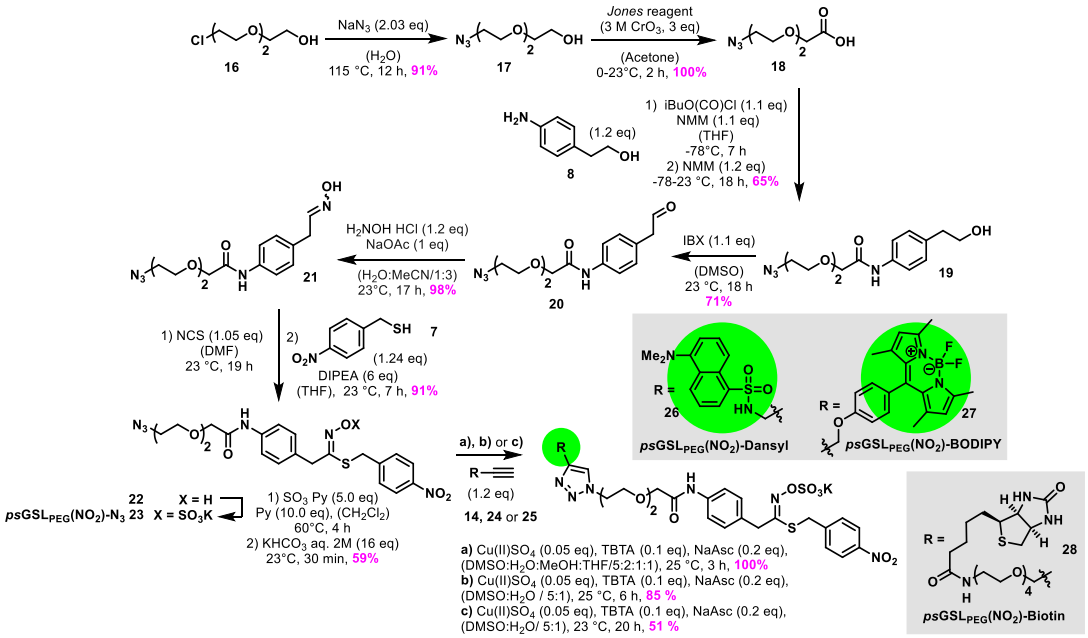
Following our initial proposal, we started to develop potentially nitro-reductase responsive *pseudo*-GSL (psGSL) probes. Therefore, the benzylthiol **7** (Scheme 2) was efficiently prepared from commercially available p-nitrobenzylalcohol **4** in a three-step sequence of *Appel* reaction, substitution of the bromide **5** with potassium thioacetat and final cleavage of the thioacetate **6** in the presence of methanolic HCl. In parallel, the oxime **11** was synthesized from the aniline **8** via a three-step synthesis including diazotation and nucleophilic substitution of the intermediate diazonium salt in the presence of sodium azide, subsequent oxidation of the azido alcohol **9**[4] and final oxime formation from the aldehyde **10**. Using compound **7** and **11**, the formation of the thiohydroximate **12** and thiohydroximate-O-sulfonate **13** was achieved following our original procedure.[1] However, after final click reaction with the BODIPY-alkyne **14**, we realized that the resulting *ps*GSL(NO_2)-BODIPY **15** was not soluble in water and therefore not usable within an aqueous enzymatic assay. As our approaches to enable water solubility through generation of carboxylate bearing *p*-nitrobenzylthiol building blocks as present in structure **1** failed due to difficulties in the synthesis, we decided to improve water solubility by incorporation of a short PEG chain into the design of the probe.[5] Starting from commercially available PEG-chloro alcohol **16** (Scheme 3), substitution with sodium azide led to the formation of PEG-azido alcohol **17**[6], which was oxidized to its corresponding acid **18**[7] in the presence of *Jones* reagent and coupled to the aniline **8** after activation with isobutyl chloro formiate.



Scheme 2: Synthesis of ***ps*GSL(NO$_2$)-BODIPY**.

Similar as before, the resulting alcohol **19** was oxidized to the aldehyde **20** and finally converted to the oxime **21**, from which the corresponding thiohydroximate-O-sulfonate **23** was obtained via the original synthesis procedure.[1] Based on **23** the fluorescent probes *ps*GSL$_{PEG}$(NO$_2$)-Dansyl **26** and *ps*GSL$_{PEG}$(NO$_2$)-Dansyl **27** as well as the biotin labelled probe *ps*GSL$_{PEG}$(NO$_2$)-Biotin **28** were obtained by copper-mediated click reaction. All these probes showed appropriate water solubility for futher enzymatic investigations.[8]

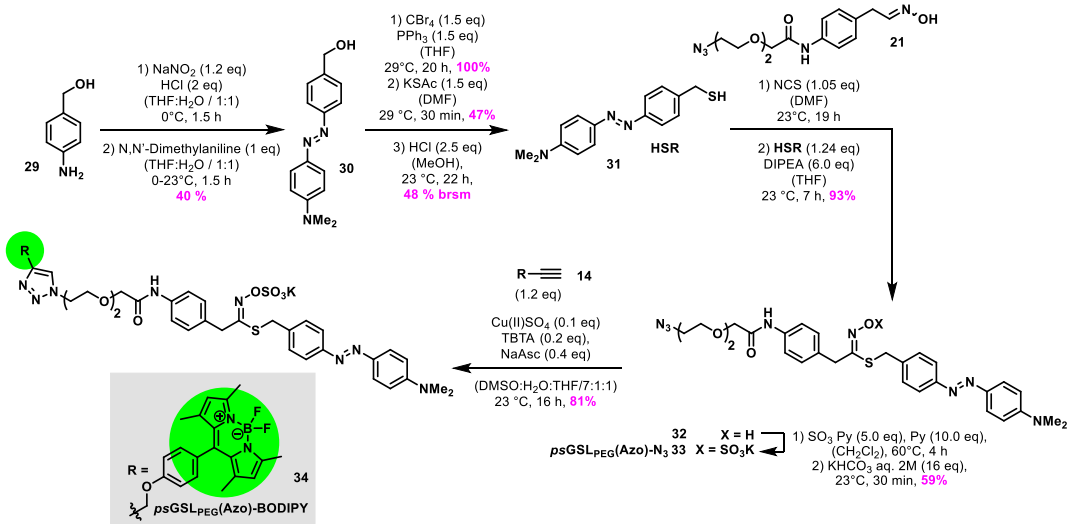
To our delight, our initial biochemical evaluations of the probes were successful and we have been able to demonstrate by LS-MS analysis that the psGSL probes **23**, **26** and **27** (Scheme 3) indeed are cleaved in the presence of nitroreductase NTR from *E. coli* releasing their corresponding ITCs. Furthermore, we were able to isolate fluorescently labelled NTR by SDS Gel, which is currently investigated by LC-HRMS analysis after enzymatic digest and in whole protein analysis in collaboration with partners inorder to determine the labelling position. In addition, we have been able to identify the cleavage and release of corresponding ITCs also in presence of NTR producing whole cell bacteria. However, further investigations are ongoing to understand and exploit the observed results.[8]



Scheme 3: Synthesis of ***ps*GSL$_{PEG}$(NO$_2$)-N$_3$** and derived probes.

In parallel, we synthesized the thiol building block **31**[9] (Scheme 4) bearing an azo-benzene moiety in order to construct potentially azo-reductase responsive *ps*GSL probes. Therefore, the diazo-coupling of

the intermediate diazonium salt obtained from **29** with N,N-dimethylaniline gave the alcohol **30**, which was converted into compound **31** by the earlier successful sequence of *Appel* reaction, nucleophilic substitution with potassium thioacetate and final acetate cleavage in the presence of methanolic HCl. Having **31** in hands, the *ps*GSL$_{PEG}$(Azo)-N$_3$ **33** was obtained utilizing oxime **21** and using our standard protocols.[1] Finally, *ps*GSL$_{PEG}$(Azo)-BODIPY **34** was generated by copper-mediated click reaction with BODIPY-alkyne **14**. This probe showed similarly to the probes **26**-**28** good water solubility.[8]



Scheme 4: Synthesis of ***ps*GSL$_{PEG}$(Azo)-BODIPY**.

In order to biochemically investigate the enzymatic cleavage of probe **34**, we recombinantly expressed Azo-R, an azo-reductase from *E. coli*, and purified the enzyme in collaboration with Prof. Schallmey from the Institute of Biochemistry at TU Braunschweig. To our delight, we have been able to demonstrate by LS-MS analysis and fluorescence emission spectroscopy that the psGSL probes **33** and **34** (Scheme 3) indeed undergo a fluorescence turn-on in the presence of azo reductase Azo-R from *E. coli* releasing their corresponding fluorescent ITCs. In addition, fluorescence turn-on has also been observed upon incubation of the probes with different Azo-R producing bacteria. However, further investigations are ongoing to understand and exploit the observed results.[8]

3. Conclusion

In summary, we were able to generate several nitro- and azo-reductase responsive *ps*GSL probes including Turn-on fluorophores. Their initial biochemical evaluation showed the release of corresponding ITCs in the presence of the enzymes and additionally cleavage was also observed in the presence of enzyme producing bacterial strains.

4. Literature

[1] C. P. Glindemann, A. Backenköhler, M. Strieker, U. Wittstock, P. Klahn, *ChemBioChem* **2019**, *19*, 1668–1694.
[2] P. Klahn, M. Brönstrup, V. Fetz, S. Hüttel, K. I. Mohr, W. Tegge, W. Collisi, *New Targeted Cytotoxic Ratjadone Derivatives and Conjugates Thereof*, **2019**, WO2019/03028431 A1.
[3] P. Klahn, V. Fetz, A. Ritter, W. Collisi, B. Hinkelmann, T. Arnold, W. Tegge, K. Rox, S. Hüttel, K. I. Mohr, et al., *Chem. Sci.* **2019**, *10*, 5187–5425.
[4] N. Dubey, P. Sharma, A. Kumar, *Synth. Commun.* **2015**, *45*, 2608–2626.
[5] K. Knop, R. Hoogenboom, D. Fischer, U. S. Schubert, *Angew. Chem. Int. Ed.* **2010**, *49*, 6288–6308.
[6] M. Rauschenberg, E. C. Fritz, C. Schulz, T. Kaufmann, B. J. Ravoo, *Beilstein J. Org. Chem.* **2014**, *10*, 1354–1364.
[7] G. Clavé, H. Boutal, A. Hoang, F. Perraut, H. Volland, P. Y. Renard, A. Romieu, *Org. Biomol. Chem.* **2008**, *6*, 3065–3078.
[8] C. C. Jimidar, L. Wiese, M. Stirtz, A. Schallmey, U. Beutling, MarkBrönstrup, P. Klahn, *unpublished results* **2021**.
[9] S. Chng, E. M. Parker, J. P. Griffiths, M. G. Moloney, L. Y. L. Wu, *Appl. Surf. Sci.* **2017**, *401*, 181–189.