Synthesis of Strained Ring Systems for Bioorthogonal Labeling Applications

summary of the dissertation

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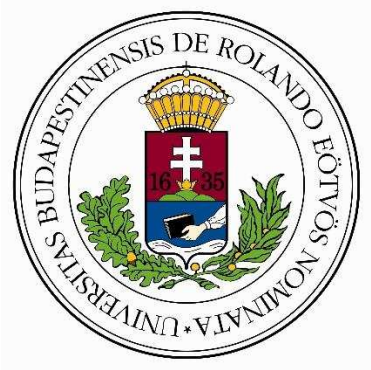

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Introduction

Imaging techniques used to visualize subcellular structures and dynamic interactions of biomolecules play an increasing role in modern molecular biology. Until recently, the most prominent techniques were those based on green fluorescent protein (GFP) and its derivatives, the discovery for which the Nobel Prize was awarded in 2008. Although the merits of the GFP related discoveries and techniques are indisputable it cannot be used for imaging biomolecules that are not directly encoded in the genome (e.g. lipids, nucleotides, saccharides). Post-translational modifications of proteins are also impossible using GFPs. In such cases the use of exogenously delivered probes are the method of choice. It is crucial that these synthetic imaging agents do not alter the function, structure and fate of the labeled biomolecule and the chemical transformation used to covalently implement these probes to the target biomatter is chemoselective, high yielding and proceed within a reasonable time domain. At the same time neither the reagents nor the products are toxic. Chemical transformations meeting all these criteria were collected under the term bioorthogonal.

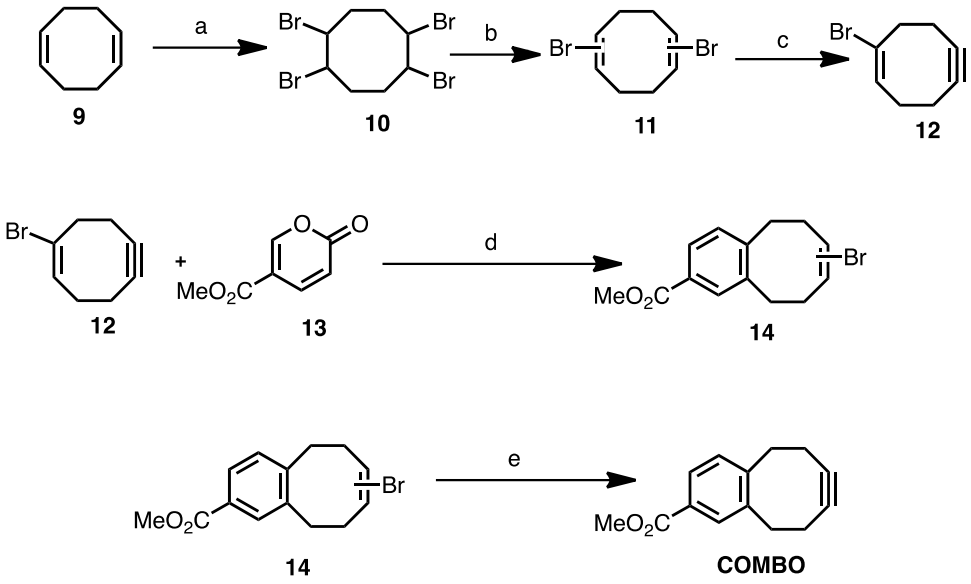
In other words, bioorthogonal functional groups are non-toxic, biologically inert (bio), and they react selectively and quasi quantitatively with their complementary function (orthogonal) at speeds comparable with that of biochemical reactions. Quite understandably, there are only a few reactions that can fulfill these very strict conditions. Among bioorthogonal transformations probably the 1,3-dipolar cycloaddition of azides with cyclooctynes and the inverse electron demand Diels-Alder reaction of tetrazines and trans cyclooctenes are the most valuable ones.

Goals

The goal of our work was to make improvements to these two methods by developing new reagents. First this was accomplished by the introduction of a novel cyclooctyne termed COMBO, which displays good kinetics and has a relatively low lipophilicity compared to reactants within the same kinetic range. Then we aimed at synthetizing new trans cyclooctenes with low lipophilicity to minimize undesired non-specific secondary interactions with the cell membranes and other subcellular structures.

Synthesis and properties of COMBO

We have devised a route where the synthesis of carboxymethyl-monobenzocyclooctyne, **COMBO** (2-methoxycarbonyl-5,6,9,10-tetrahydro-7,8-dehydro-benzocyclooctene) was aimed. It is noteworthy that COMBO lacks fluorine substituents and carry a carboxylic function that allows for further modification (**Scheme 1.**). The reaction scheme includes key intermediate (E)-1-bromocyclooct-1-en-5-yne (**12**)[1], which was hoped to react smoothly with electron deficient dienes in an inverse electron demand Dield-Alder (iEDDA) reaction.



Scheme 1. Synthesis of carboxy-monobenzocyclooctyne. a) Br_2, CH_2Cl_2, 0 °C, 85%; b) tBuOK, Et_2O, r.t., 83%; c) tBuOK, 18-crown-6 ether, hexane, r.t. d) r.t. 36% for two steps; e) tBuOK, 18-crown-6 ether, hexane, 58 °C, 47%.

Investigation of the stability of COMBO

We have further investigated the stability of COMBO by monitoring its ^{1}H NMR spectra under various conditions: (25±0.1) °C in CD_3CN:D_2O 3:1 v/v for 44 h, then at (37±0.1) °C in CD_3CN for 9 h. A potential background reaction with a biologically relevant reagent was studied at (25±0.1) °C in CD_3CN:D_2O 1:1 v/v with three-fold molar excess of glutathione (15 mM COMBO and 45 mM glutathione) for 14 h. While there was no significant change in CD_3CN:D_2O 3:1 v/v after 44 h (ca. 5% decomposition), approximately 9% of COMBO reacted in the presence of glutathione after 14 h. We observed considerable decomposition (ca. 30% after 9

h) of COMBO in acetonitrile at 37 °C, but interestingly no decomposition in the first two hours of the experiment.

Kinetic measurements

The results of the kinetic measurements have indicated a second order rate constant of $k_2 = (0.235 \pm 0.006)\ M^{-1}s^{-1}$ in acetonitrile, which is quite similar to that of the fluorinated regioisomer, DIFBO[2]. As expected, the rate constant was even larger in a more polar medium ($k_2 = 0.795 \pm 0.007\ M^{-1}s^{-1}$ in water-acetonitrile 3:4 v/v). In comparison with DIFO[3] and DIBO[4] COMBO showed ca. 3 and 5 times faster reaction rates. Direct comparison with BCN[5] is not possible, as kinetic data in acetonitrile were not reported for this reagent, however, the fact that BCN shows comparable kinetics only in a much more polar medium implies the superior reactivity of COMBO.

Computational studies

As gem-diluorinated compounds usually show increased reactivity relative to their non-fluorinated derivatives, we were surprised to see that the reaction rates for DIFBO and COMBO were yet comparable. To explain the experimentally observed reaction rates we carried out density functional theory (DFT) calculations for the cycloadditions of methyl azide with DIFBO and COMBO using the B3LYP functional as well as the 6-311++G** basis set implemented in the Gaussian 03 suite.

The HOMO and LUMO are lower in energy for DIFBO than those for COMBO, while the resulting FMO gap is somewhat narrower for COMBO. As of the ring strain, we can conclude that, in contrast to DIFBO, the equilibrium structure of COMBO is slightly more bent and closer to the transition-state of the reaction. The narrower FMO gap and the less distortion energy required to achieve the transition state are presumably the main reasons for the lower barrier height of COMBO.

Lipophilicity of COMBO.

A drawback of aromatic rings is their lipophilicity, due to their low water solubility and because they may engage in hydrophobic interactions with proteins. This makes the only one aromatic ring containing COMBO even more appealing, since as we have seen in the kinetic

studies, its reactivity is reasonably close to DIBAC and BARAC[6], while it is expected to be much less lipophilic.

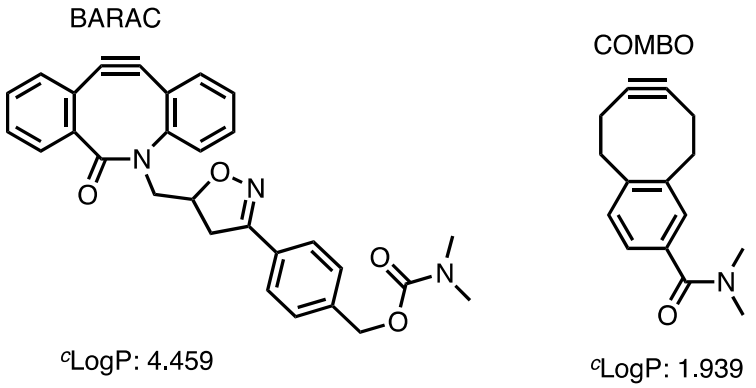


Figure 1. cLogP values of the N-methylamide derivatives of COMBO and BARAC

We calculated cLogP values using BioByte (embedded in ChemDraw Ultra 12) for the N-methylamide derivatiove of both COMBO and BARAC and indeed COMBO proved to be much less lipophilic (**Figure 1.**)

Labeling of azido-glycoproteins on HeLa Cells and Imaging by Fluorescence Microscopy

Next, we wanted to see the applicability of fluorescently tagged COMBO reagents in live cell staining experiments. For this, COMBO-acid was conjugated to a fluorescein-piperazine derivative[7] by means of amide bond formation to furnish COMBO-Flu. Live cell imaging was performed on HeLa cells metabolically modified with Ac_4ManNAz on their glycan structures (see supporting information)[8]. The cells were stained with 12.5 μM COMBO-Flu. The cells incorporate Ac_4ManNAz and express it as azidosialic acid in their glycoproteins at the trans-Golgi network, which are then targeted mainly to the plasmamembrane. The cells showed efficient azide specific labeling with no significant background fluorescence. The live staining appeared efficient at 5 μM concentration for 1 h, too.

Synthesis and properties of novel trans cyclooctenes

The introduction of the TCO-tetrazine based cycloadditions by the Fox group in 2008[9] was a major breakthrough in bioorthogonal chemistry. The good stability of the reaction partners and the excellent kinetics made these reagents an ideal tool for bioorthogonal labeling experiments. The initial method used a dipyridyl-tetrazine derivative with a second order rate constant of $k_2 = (2000 \pm 400)$ $dm^3mol^{-1}s^{-1}$ at 25 °C in 9:1 methanol/water. The Hilderbrand group introduced a novel asymmetric benzylamino tetrazine derivative, which showed even better kinetics with trans cyclooctenol (TCO-OH, $k_2 = (33585 \pm 326)$ $dm^3mol^{-1}s^{-1}$ in PBS at 37 °C)[10] and an excellent stability (15% decomposition observed after 15 h in fetal bovine serum (FBS) at 20.0 ºC)[11,12]. The past few years saw numerous applications of this chemistry[13,14,15,16,17] and attempts to optimize the tetrazine reactivity/stability relationship[18,19].

The major problem encountered in these applications came from the high lipophilicity of trans cyclooctenol (TCO-OH), an 8 membered carbon ring, lacking any other polar substituents than the OH used for conjugation. Lipohilicity increases undesired nonspecific binding and so it became important to develop TCO derivatives with decreased lipophilicity. Also as investigations in tetrazine chemistry did not lead to new reagents that would have more favorable reactivity/stability relationship, it became more important to look into the cyclooctene chemistry to improve reaction kinetics. We envisioned that trans cyclooctenes incorporating an endocyclic acetal moiety would allow both more favorable kinetics and better solubility.

Synthesis of DO-TCO and EG-TCO.

Jendralla[20] already reported a synthesis for trans cyclooctenes incorporating an endocyclic acetal moiety. Our synthesis which is a modification of Jendralla's method, started from commercially available dioxepin cis-4,7-dihydro-1,3-dioxepin in order to yield **DO-TCO (22, Figure 2.)**, a bishetero trans cyclooctene bearing an OH handle for conjugation.

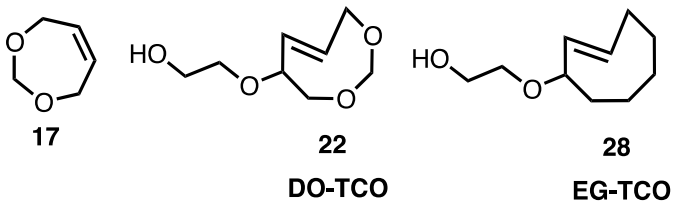
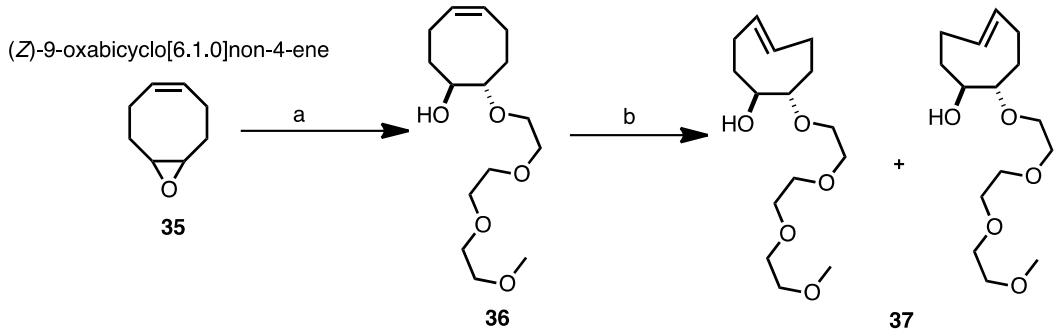


Figure 2. cis-4,7-dihydro-1,3-dioxepin, DO-TCO and EG-TCO

Kinetic studies of DO-TCO in PBS at 37 °C gave a second order rate constant of $k_2 = (332 \pm 3)$ dm^3 mol^{-1} s^{-1} for the reaction with benzylamino tetrazine which is two orders of magnitude lower than the values measured for TCO. This result is surprising, given that DO-TCO is expected to have much larger internal strain than TCO. In order to examine if this lower reactivity is caused by the incorporation of endocyclic oxygens or by the ether group adjacent to the trans double bond, we decided to synthetize **EG-TCO** (**28, Figure 2.**), which is a direct analog of DO-TCO . Surprisingly, the second order rate constant for **EG-TCO** proved to be only $k_2 = (600 \pm 6)$ dm^3 mol^{-1} s^{-1} in PBS at 37 °C. While it is still fifty times lower than the second order rate constant for TCO-OH, it is twice of the value measured for DO-TCO. Clearly, these values indicate that the relatively low reactivity of EG-TCO and DO-TCO in the inverse electron demand Diels-Alder reaction with benzyl amino tetrazine is to a great degree a result of steric hindrance exerted by the nearby ether group. This fact alone though does not explain the even lower kinetics of the more strained DO-TCO compared to EG-TCO. Literature studies show that in general there is considerable rate acceleration when aqueous solutions are used as media in Diels-Alder reactions[21]. It seems that this acceleration is due to the so-called hydrophobic effect, which would be less pronounced for the polar DO-TCO. This proposal, however, needs to be further studied in solvent dependent kinetic experiments.

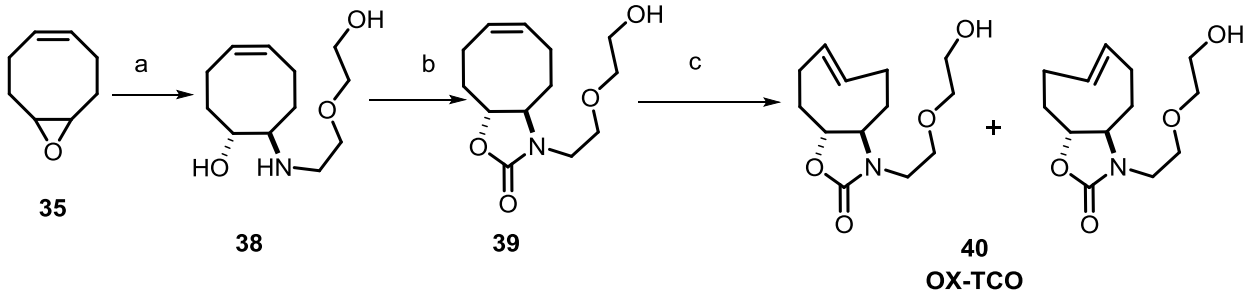
Synthesis of 3PEGMe-TCO and OX-TCO.

Since our attempts to increase reactivity through the introduction of extra ring strain and to decrease lipophilicity through the incorporation of polar moieties into the ring had failed, we designed trans cyclooctenes 3PEGMe-TCO (**37**) and OX-TCO (**40**) (**Scheme 3.** and **Scheme 4.**).



Scheme 3. Synthesis of 3PEGMe-TCO. a) 3PEGMe, $Er(OTf)_3$, r.t., 41%; b) hν, methyl benzoate, 4:1 Et_2O/hexanes, 64%.

These have no additional ring strain yet bear highly polar substituents but not too close to the reaction center. We had a reason to think that this would diminish the "hydrophobic effect" in aqueous media less than endocyclic polar moieties.



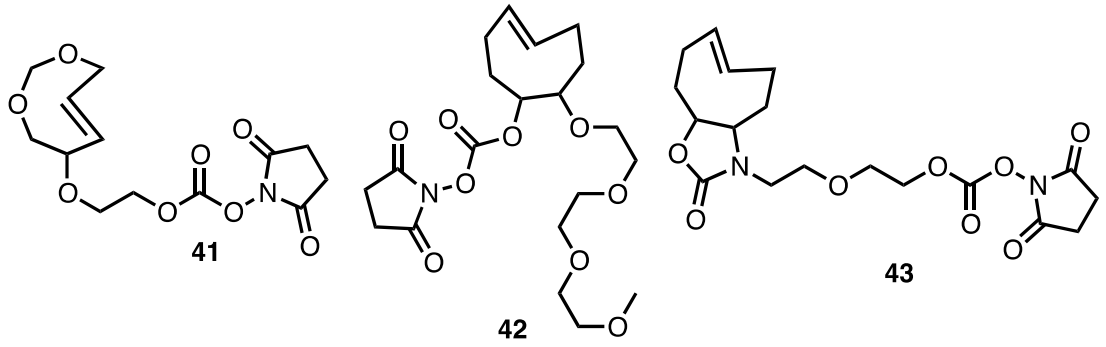
Scheme 4. Synthesis of OX-TCO. a) 2-(2-aminoethoxy) ethanol, MW, 130 °C, 85%; b) N,N′-disuccinimidyl carbonate, Et_3N, acetonitrile, r.t., 92%; c) hν, methyl benzoate, 1 vol% MeOH in Et_2O, 82%.

Synthesis of NHS carbamates of DO-TCO, 3PEGMe-TCO and OX-TCO.

Next we prepared NHS carbamates from DO-TCO, 3PEGMe-TCO and OX-TCO (**Scheme 5.**), using *N,N′*-disuccinimidyl carbonate as reagent in the presence of Et_3N in acetonitrile at room temperature. In the case of 3PEGMe-TCO (**37**) and OX-TCO (**40**), we used an isomeric mixture of the trans compounds for the conversion to the NHS carbamates (for simplicity only one of the isomers is shown for both compounds in **Scheme 5.**). Luckily, the two trans isomers of **42** could be isolated on silica, so we were able to perform kinetic measurements for both isomers of **37**, while in the case of **40** we had to measure kinetics on the 4:1 mixture of isomers. We did not identify which of the two possible structures of **37** correspond to the first and to the second

isomer isolated. Nevertheless, the two second order rate constants proved to be $k_2 = (20643 \pm 387)$ dm^{3}mol^{-1}s^{-1}for the first isomer and $k_2 = (108041 \pm 2418)$ dm^{3}mol^{-1}s^{-1} for the second in PBS at 37 °C for their reaction with benzylamino tetrazine.

This difference in rate constants for the two isomers may seem unexpected, however, literature examples show that there can be even up to one order of magnitude difference in the reactivities of different isomers of TCOs in reactions with tetrazines depending on the position (axial or equatorial) of the substituent opposite to the trans double bond[22]. The isomer mixture of **40** gave



Scheme 5. The NHS carbamates prepared from our TCOs.

a second order rate constant of $k_2 = (29242 \pm 636)$ dm^3 mol^{-1} s^{-1} under the same conditions. This aligns well with the reactivity of TCO-OH.

Comparison of ^{c}LogP values for our and previously reported TCOs

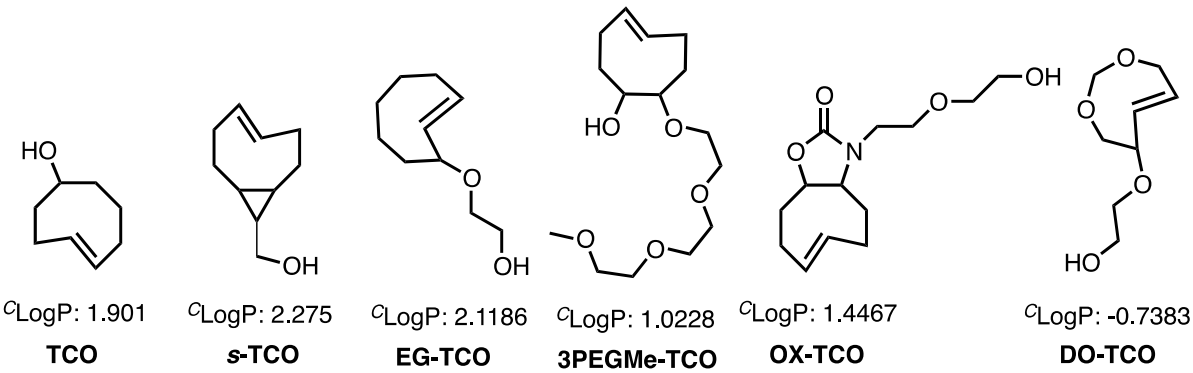


Figure 2. ^{c}Log P values of previously reported and our trans cyclooctenes as calculated by BioByte embedded in CS ChemDraw Ultra 12.

Figure 2. shows calculated ^{c}Log P values for TCO-OH, s-TCO and our trans-cyclooctene derivatives. It can be seen that adding a glycol moiety (EG-TCO) almost does not affect the ^{c}Log

P value at all, while a longer polar chain can make a significant difference. Incorporation of oxygen into the cyclooctene ring itself shifts the ^{c}Log P drastically.

Synthesis of PARP1 inhibitor conjugates.

In order to assess the potential use of the new TCO's (DO-TCO, 3PEGMe-TCO and OX-TCO), we decided to perform imaging experiments using a previously studied model system. We choose PARP1 imaging using a PARP inhibitor, AZD2281. It has been shown that the 4-NH-piperazine of AZD2281 tolerates a diverse range of capping groups without significantly decreasing PARP1 binding affinity[23]. A previous study reported AZD2281-TCO, which is a modification of AZD2281 using the NH-piperazine anchor point. AZD2281-TCO was then used in live-cell imaging with fluorophore-tetrazine derivatives[24]. Similarly, starting from the NHS-carbamates we prepared AZD2281-DO-TCO, AZD2281-3PEGMe-TCO and AZD2281-OX-TCO and the originally reported AZD2281-TCO in order to compare their performance in in vivo imaging experiments. A significant advantage of the new trans cyclooctene derivatives lies in their better solubility in DMSO/water mixtures. Sample preparation using our compounds was significantly easier than with AZD2281-TCO. Bioorthogonal imaging experiments of PARP protein in HT1080 cells (expressing PARP1 fused to mCherry) were successful with all three new compounds, but we could not yet observe a significant difference between AZD2281-TCO and our products. Further experiments would be needed to assess the potential of DO-TCO, 3PEGMe-TCO and OX-TCO.

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[1] Detert, H.; Rose, B.; Mayer, W.; Meier, H. Chem. Ber. **1994**, 127, 1529-1532.

[2] Sletten, E. M.; Nakamura, H.; Jewett, J. C.; Bertozzi, C. R. J Am Chem Soc. **2010**, 9, 11799-11805.

[3] Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. Proc. Natl. Acad. Sci. U.S.A. **2007**, 104, 16793–16797

[4] Sanders, B. C.; Friscourt, F.; Ledin, P. A.; Mbua, N. E.; Arumugam, S.; Guo, J.; Boltje, T. J.; Popik, V. V.; Boons, G-J. J. Am. Chem. Soc. **2011**, 133, 949-957.

[5] Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J. T.; van Hest, J. C. M.; Lefber, D. J.; Friedl, P.; van Delft F. L. Angew. Chem. Int. Ed. **2010**, 49, 9422-9425.

[6] Debets, M.; Berkel, S. van; Dommerholt, J.; Dirks, A.; Rutjes, F.; van Delft, F. Acc. Chem. Res. **2011**, 44, 805–815.

[7] Chang, P. V.; Prescher, J. A.; Hangauer, M. J.; Bertozzi, C. R. J. Am. Chem. Soc. **2007**, 129, 8400-8401.

[8] V. Hong; N. F. Steinmetz; M. Manchester; M. G. Finn; Bioconjugate Chem. **2010**, 21, 1912-1916

[9] Blackman, M. L.; Royzen M.; Fox, J. M. J. Am. Chem. Soc. **2008**, 130, 13518-13519.

[10] Own data.

[11] Devaraj, N. K.; Upadhyay, R.; Haun, J.B.; Hilderbrand, S. H.; Weissleder, R. Angew. Chem. Int. Ed. **2009**, 48, 1-5.

[12] Devaraj, N. K.; Weissleder, R; Hilderbrand, S. H. Bioconjugate Chem. **2008**, 19, 2297-2299.

[13] Rossin, R.; Verkerk, P.; Bosch, S. van den; Vulders, R.; Verel, I.; Lub, J.; Robillard, M. Angew. Chem. Int. Ed. **2010**, 49, 3375-3378.

[14] Devaraj, N. K.; Weissleder, R. Accounts Chem. Res. **2011**, 44, 816-827.

[15] Keliher, E.; Reiner, T.; Turetsky, A.; Hilderbrand, S.; Weissleder, R. ChemMedChem **2011**, 6, 424–427.

[16] Seitchik, J. L.; Peeler, J. C.; Taylor, M. T.; Blackman, M. L.; Rhoads, T. W.; Cooley, R. B.; Refakis, C.; Fox, J. M.; Mehl, R. A. JACS **2012**, 134, 2898-2901.

[17] Carlson, J.; Meimetis, L.; Hilderbrand, S.; Weissleder, R. Angew. Chem. Int. Ed. **2013**, 52, 6917-6920.

[18] Wiessler, M.; Waldeck, W.; Kliem C.; Pipkorn, R.; Braun, K. Int. J. Med. Sci. **2010**, 7, 19-28.

[19] Karver, M. R.; Weissleder, R.; Hilderbrand, S. A. Bioconjugate Chem. **2011**, 22, 2263-2270.

[20] Jendralla, H. Chem. Ber. **1982**, 115, 201-209.

[21] Wijnen, J.W.; Zavarise, S.; Enghberts, F.N. J. Org. Chem. **1996**, 61, 2001-2005.

[22] Rossin, R.; van den Bosch, S. M.; Ten Hoeve, W.; Carvelli, M.; Versteegen, R. M.; Lub, J. Bioconjugate Chem. **2013**, 24, 1210−1217.

[23] Menear, K. A.; Adcock, C.; Boulter, R.; Cockcroft, X.-I.; Copsey, L.; Cranston, A.; Dillon, K. J.; Drzewiecki, J.; Garman, S.; Gomez, S.; Javaid, H.; Kerrigan, F.; Knights, C.; Lau, A.; Loh, V. M., Jr.; Matthews, I. T. W.; Moore, S.; O'Connor, M. J.; Smith, G. C. M.; Martin, N. M. B. J. Med. Chem. **2008**, 51, 6581-6591.

[24] Reiner, T.; Earley, S.; Turetsky, A.; Weissleder, R. Chembiochem **2010**, 11, 2374-2377.