Masked Amino Trimethyl Lock (H$_2$N-TML) Systems: New Molecular Entities for the Development of Turn-On Fluorophores and Their Application in Hydrogen Sulfide (H$_2$S) Imaging in Human Cells

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Abstract: Masked trimethyl lock (TML) systems as molecular moieties enabling the bioresponsive release of compounds or dyes in a controlled temporal and spatial manner have been widely applied for the development of drug conjugates, prodrugs or molecular imaging tools. Herein, we report the development of a novel amino trimethyl lock (H$_2$N-TML) system as an auto-immolative molecular entity for the release of fluorophores. We designed Cou-TML-N$_3$ and MURh-TML-N$_3$, two azide-masked turn-on fluorophores. The latter was demonstrated to selectively release fluorescent MURh in the presence of physiological concentrations of the redox-signaling molecule H$_2$S in vitro and was successfully applied to image H$_2$S in human cells.
bioresponsive smart materials, modern drugs or cell imaging.[1,2] Considering the tremendous success of classical masked TMLs for molecular release systems, we envisaged that novel masked amino trimethyl lock (H$_2$N-TML) systems based on ortho-amino dihydro cinnamic acid derivatives could further broaden the scope of enzymes or chemical triggers applicable for the release of compounds.

To prove our hypothesis that ortho-amino dihydro cinnamic acid derivatives can serve as auto-immolative moieties for molecular release of compounds, we planned to synthesize morpholino urea rhodamine (MURh) N-acylated with masked H$_2$N-TML moieties (A, Scheme 2). N-Acylation of this dye is known to quench its fluorescence,[18–22] thus resulting in a turn-on fluorophore, releasing the parent fluorophore MURh upon chemical or enzymatic demasking towards the free H$_2$N-TML-MURh and auto-immolative lactamization of the H$_2$N-TML moiety under release of dihydroquinolin-2(1H)-one 1.

For proof of principle of this concept, we synthesized N$_3$-TML-CO$_2$H (Scheme 3) representing an H$_2$N-TML masked with an azido moiety, which was supposed to release the corresponding H$_2$N-TML upon Staudinger reduction of azide. Starting from commercially available 3,3-dimethyl acrylic acid (2) and 3,5-dimethylaniline (3), amide 4 was formed in the presence of thionyl chloride and triethylamine at 80°C in 91% yield, which could be converted by an intramolecular Friedel-Crafts alkylation in the presence of aluminum trichloride into the dihydroquinolin-2(1H)-one 1 in 97% yield. The installation of a tert-butyl carbamoyl moiety at the nitrogen led to formation of compound 5, which allowed the saponification of the amide in the presence of aqueous lithium hydroxide solution to obtain the

Scheme 2. Principle of masking and molecular release of amino trimethyl lock (H$_2$N-TML) turn-on fluorescent dyes. PG refers to a protective group (e.g. –NO$_2$, –N$_3$, peptides or azo benzenes), masking H$_2$N-TML's lactamization reactivity.

Scheme 3. Synthesis of N$_3$-TML-CO$_2$H. Abbreviations: Im = 1H-imidazole, xs = excess of the reagent.
N-Boc-protected H₂N-TML-CO₂H 6 in quantitative yield over both steps. First evidence for a fast and clean lactamization of the free H₂N-TML-CO₂H was found by the quantitative conversion of 6 into dihydroquinolinol-2(1H)-one 1 within less than 1 min in the presence of an excess of TFA in dichloromethane. The carboxylic acid moiety of 6 was then activated in the presence of ethyl chloroformate and triethyl amine and subsequently reduced with sodium borohydride in the presence of methanol to obtain the N-Boc-protected H₂N-TML-alcohol 7 in a moderate yield of 57%. Different silyl protection groups were installed, however, only TBDPS was stable enough to allow a selective deprotection of the Boc group in the presence of 1.0 M HCl in dry EtOAc, yielding the anilinium chloride 11 in 72% yield. A diazotation of the aniline with trifyl azide in the presence of catalytic amounts of copper(II) sulfate gave the azide 12 in 74% yield. Alternatively, the Goddard-Borger reagent,[23] a bench stable and safe diazotation reagent, led to slightly decreased yield of 65%. Finally, deprotection of the TBDPS group in the presence of TBAF and subsequent one-pot oxidation in the presence of sodium hypochlorite, sodium chlorite and catalytic amounts of TEMPO led to the formation of the desired N₃-TML-CO₂H in 20% overall yield over 10 steps.

With N₃-TML-CO₂H in hand, we synthesized N₃-TML-MURh (Scheme 4) by N-acylation of MURh in the presence of EDCI and pyridine as a first turn-on fluorophore, which should release the parent MURh upon Staudinger reduction of the azide moiety and subsequent auto-immolative lactamization. In similar manner, N₃-TML-Cou (Scheme 4) was generated by N-acylating 7-amino-4-methylcoumarine (Cou). N₃-TML-Cou showed only very weak blue fluorescence at an excitation wavelength of λₑₓ = 366 nm, while N₃-TML-MURh was practically non-fluorescent (Scheme 5). When both compounds, N₃-TML-Cou and N₃-TML-MURh, were reacted under Staudinger reduction conditions in the presence of triphenylphosphine and acetic acid in a THF/water mixture, the occurrence of a strong blue and green

Scheme 4. Synthesis of N₃-TML-MURh and N₃-TML-Cou. Abbreviations: xs = excess of the reagent.

Scheme 5. Proof of principle for the turn-on fluorophores N₃-TML-Cou and N₃-TML-MURh: Release of the fluorophores Cou and MURh in the presence of PPh₃ or TCEP. UV = Excitation at 366 nm. Pictures show response under condition a).
fluorescence, respectively, was observed within the first 2 min after addition of the triphenylphosphine, clearly indicating the release of the parent fluorophores (Scheme 5). Both parent dyes Cou and MURh as well as the dihydroquinolin-2(1H)-one 1 have been isolated from the reaction mixtures and identified by TLC-MS as well as $^1$H NMR.

In addition, when $N_3$-TML-Cou and $N_3$-TML-MURh were dissolved in PBS buffer at pH 7.4, the release of the parent fluorophores could also be achieved in the presence of the water-soluble tris(2-carboxyethyl)phosphine (TCEP) within 2 min at 23°C (see Supporting Information Figure S4).

Observing the fast fluorescence turn on during the conversion of $N_3$-TML-Cou and $N_3$-TML-MURh and considering the short reaction time for lactamization of 6 (Scheme 3) under acidic and aqueous conditions, we applied systematic conformational analysis and intrinsic reaction path following techniques at the density functional level of theory ($\omega$B97XD/6-311+$^+$G*$$/\omega$B97XD/6-31G*) using an implicit water solvent model in order to predict the relative rate constants for both the TML lactonization and the lactam ring closures. As early as 1987 Houk and co-workers applied Hartree-Fock theory and limited molecular models, namely isolated hydroxy acids in the gas phase, in order to develop the relevant force field parameter, which the authors in a second step used to study a variety of different hydroxy acids. Adapting those molecular models in 2009, Karaman used semiempirical AM1 and empirical MM2 calculations to study the thermodynamic and kinetic parameters for pharmaceutically relevant trimethyl lock systems in hydroxy hydro cinnamic acids. Many of these earlier studies were hampered by the fact that, based on both inevitably simplified wave functions and the underlying molecular models, a lot of assumptions concerning the real structure and energy of the transition state had to be made. Houk and co-workers simply assumed that the Hartree-Fock/3-21G results, especially the predicted O to C distance (2.05 Å) of the “tetrahedral complex” in the gas phase, were similar to that of the transition state in solution.

In our computational study we therefore focused on the unique localization of the relevant transition structures in order to predict the relative rate constants of both lactonization and lactamization reactions, that means the transition structures of the ring closures, if we assume this step to be rate determining.

Starting with an OH to C=O distance of 2.05 Å as proposed by the studies of Houk and Karaman, but applying DFT theory embedded in a standard continuum solvent model, we nevertheless could not localize any transition structure at all. Even in the case of a protonated carbonic acid, the reaction path is repulsive, preventing any tetrahedral intermediate. Due to our DFT simulations the esterification and lactamization go through a real transition state (Figure 1, A) only if the process is modeled as synchronous C—O bond formation in combination with a proton abstraction (Please see animation: C-O.gif in Supporting Information). Note the high activation barrier of around 40 kcal/mol. B: The analogue IRC path of the lactamization (again, without TML, see animation CN.gif in Supporting Information). C: Reaction path of lactonization with proton shuttle: an additional carboxylic moiety, simplified as a formic acid, was included in our simulation. The activation barrier is low (around 15 kcal/mol). D: Reaction path of the analogue lactamization including formic acid as a proton shuttle with an even lower barrier of 10 kcal/mol.

![Figure 1](https://example.com/figure1.png)
changes in the mono-molecular mechanism. The proton shuttle mechanism simulation model much more realistic in comparison with the reduced to a value below 12 kcal/mol (Figure 1, C), making our investigated whether the release of the parent fluorophores (Figure 2). While

H$_2$S is an important gaseous signaling molecule playing numerous roles in healthy metabolism of human, bacterial and plant cells and is involved in several physiological processes such as the regulation of the intracellular redox status, neuronal transmission or the relaxation of smooth muscles, but also plays a central role in the pathophysiology of different diseases. Therefore, cellular detection of H$_2$S by fluorescent probes is of high interest and azide-based probes for this purpose have been described earlier. However, an important criterion for the proper imaging of H$_2$S levels is a good selectivity of the probe for the detection of H$_2$S in the presence of multiple other biological sulfur species such as cysteine, glutathione (GSH), or lipoic acid (LA). Therefore, we explored the selectivity of both probes in the presence of a variety of different reactive sulfur and nitrogen species known to be present in human tissue (Figure 3). Despite the relatively high fluorescence background, N$_2$-TML-Cou displayed a significant response to H$_2$S, cysteine and glutathione, while its response to lipoic acid, nitrite, thiosulfate, dithionite, thiocyanate and sulfite was less pronounced (Figure 3A). To our delight, N$_2$-TML-MURh showed a very selective response to H$_2$S.

Figure 2. A) Relative intensity of the fluorescence emission spectrum of N$_2$-TML-Cou (10 μM) in aqueous PBS buffer (50 mM, pH = 7.4 with 0.2% DMSO) after treatment with Na$_2$S (100 μM, 50 mM PBS buffer pH = 7.4) at 37 °C at different time points. B) Relative intensity of the fluorescence emission spectrum of N$_2$-TML-MURh (10 μM) in aqueous PBS buffer (50 mM, pH = 7.4 with 0.2% DMSO) after treatment with Na$_2$S (100 μM, 50 mM PBS buffer pH = 7.4) at 37 °C at different time points.

However, we were delighted to see that both N$_2$-TML-Cou and N$_2$-TML-MURh responded to physiological conditions of hydrogen sulfide (H$_2$S) by the release of their parent fluorophore (Figure 2). While N$_2$-TML-Cou was reacting much faster in the presence of H$_2$S at 100 μM concentration, reaching the maximum fluorescence emission after 60 min (Figure 2A), N$_2$-TML-MURh showed an overall slower conversion and release of the parent fluorophore MURh under the same conditions (Figure 2B).

In order simulate the condensed phase is a more realistic way, and adapting the well-known property of carboxylic acids to form dimers, we, in a second step, included one additional carboxylic moiety (simplified as a formic acid) in our molecular computer models. Due to our calculations the second hydroxy acid moiety plays the key role in both the lactone and the lactam cyclization, namely as a proton shuttle.

Starting with our dimeric hydroxy-acid educt (O to C distance: 2.42 Å), while perfectly pre-organized (“proximity effect”), showing only weak, non-covalent O–C interactions (Force constant: ± 0.4 N/cm), the protonation of the carboxyl oxygen is connected with a pronounced “shoulder” visible in the reaction coordinate. Nevertheless, only after the onset of the hydroxy deprotonation, the “true” transition state connected with a forming C–O bond (O–C force constant: ± 1.6 N/cm) is reached (please see animation: Lacton.gif in the Supporting Information). Though, due to our calculations this animation for the amino case: C-N-protonated.gif in the Supporting Information). Though, due to our calculations this...
while the presence of all other reactive sulfur and nitrogen species led to negligible fluorescence.

However, the significantly better selectivity of $\text{N}_3$-TML-MURh comes at the cost of a much higher detection limit compared to $\text{N}_3$-TML-Cou. While the H$_2$S detection limit of $\text{N}_3$-TML-MURh was determined to be 100 μM in vitro (Figure S7 in the Supporting Information), $\text{N}_3$-TML-Cou gave already a fluorescent response at 0.1 μM concentration of H$_2$S. However, as normal physiological concentrations of H$_2$S in healthy tissue and serum cover ranges between 30–300 μM and diseases associated concentrations can reach up to 600 μM,[36] less sensitive but highly selective probes like $\text{N}_3$-TML-MURh seemed to be well suited for the detection of H$_2$S in cells. Inspired by these results, we next aimed to investigate the ability of $\text{N}_3$-TML-MURh to visualize H$_2$S in mammalian cells by fluorescence microscopy. For this purpose, adherent human embryonic kidney (HEK293T) cells were incubated with $\text{N}_3$-TML-MURh and subsequently treated with Na$_2$S (50 μM) for 45 min at 37°C (Figure 4). $\text{N}_3$-TML-MURh induced a clear fluorescent signal, which seems to show a localization at mitochondria and the endoplasmic reticulum (ER) of the cells (Figure 4B and D).[37]

This signal was sulfide-dependent, as the negative control in...
absence of Na₂S was non-fluorescent (Figure 4F and H). Likewise, when the sulfide ions were removed by pre-incubation of the cells with ZnCl₂, no fluorescent signal was detected upon treatment of the cells with N₂-TLM-MURh (Figure 4J and L). These experiments demonstrate that N₂-TLM-MURh is cell-permeable and capable to visualize H₂S in a cellular context.

In summary, we designed two turn-on fluorophores based on a novel azide-masked H₂N-TML moiety suitable to enable the release of different fluorophores upon chemical or bioresponsive demasking. Furthermore, our computations suggest that the fast auto-immolative lactamization proceeds via an alkynylamine-amine proton shuttle. N₂-TLM-MURh was demonstrated to selectively respond to physiological concentrations of the important gaseous signaling molecules H₂S by release of highly fluorescent MURh and proven suitable for H₂S imaging in an in vitro cell culture model. Compared to other non-activatable probes, the N₂-TML system offers the advantage for application to H₂S-responsive prodrug approaches, as the N₂-TML-CO·H moiety could also be utilized to mask the heteroatoms (N, O, S atoms) of drugs or other effectors. To underline the potential of masked H₂N-TML for the development of turn-on fluorophores and applications in drug delivery concepts, we are currently investigating the installation of enzymatically cleavable masking groups at the H₂N-TML moiety as well as working on the development of targeted drug conjugates bearing H₂S-responsive linkers based on N₂-TML systems.

Experimental Section

Experimental details can be found in the Supporting Information.

Author Contributions

The research was conceived by P.K. The manuscript was written by P.K., J.G. and M.B. All compounds were synthesized by C.C.J. and P.K. Supporting information were prepared by C.C.J., B.K., H.F. and P.K. Computations were planned and conducted by J.G. Fluorescence spectroscopy and SRS selectivity studies were planned by P.K. and C.C.J. and conducted by C.C.J. Fluorescence microscopy and imaging studies on cells were planned by M.B., P.K., H.F. and B.K., and conducted by B.K. All authors have given approval to the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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