Bioorthogonal Catalysis

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Progress towards Bioorthogonal Catalysis with Organometallic Compounds**

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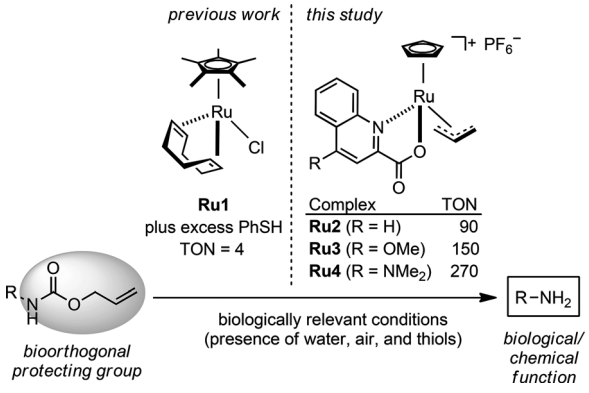
Abstract: *The catalysis of bioorthogonal transformations inside living organisms is a formidable challenge—yet bears great potential for future applications in chemical biology and medicinal chemistry. We herein disclose highly active organometallic ruthenium complexes for bioorthogonal catalysis under biologically relevant conditions and inside living cells. The catalysts uncage allyl carbamate protected amines with unprecedented high turnover numbers of up to 270 cycles in the presence of water, air, and millimolar concentrations of thiols. By live-cell imaging of HeLa cells and with the aid of a caged fluorescent probe we could reveal a rapid development of intense fluorescence within the cellular cytoplasm and therefore support the proposed bioorthogonality of the catalysts. In addition, to illustrate the manifold applications of bioorthogonal catalysis, we developed a method for catalytic in-cell activation of a caged anticancer drug, which efficiently induced apoptosis in HeLa cells.*

Bioorthogonal chemistry in living cells and animals has extended our biological understanding and, moreover, has had a profound impact on fields such as imaging, drug development, and biotechnology.[1] To date, bioorthogonal chemistry has mainly been stoichiometric as well as uncatalyzed, and only a few artificial catalytic reactions in biological environments have been reported to date.[2–6] Bioorthogonal catalysts selectively recognize specific functional groups absent in nature and catalyze their transformation within (living) biological systems. To manage this task, the catalyst needs to perform a difficult balancing act between reactivity and stability. The combined presence of water, air, and a plethora of cellular components such as millimolar concentrations of thiols demand a high stability of the catalyst, while, at the same time, the physiological conditions (e.g. temperature and pH value) and low cellular concentrations of the reactants require a high reactivity towards its designated substrate. Furthermore, neither the catalyst nor the substrate must intrinsically affect the biochemical homeostasis of the host. Even though there is a lack of efficient bioorthogonal catalysts available today that match these crucial criteria, a variety of attractive applications can be envisioned, ranging from turning over protected (caged) substrates/prodrugs to catalytically labeling or deactivating target biomolecules. The advantage of signal amplification through catalytic turnover has already been successfully exploited in the area of enzyme-triggered bioimaging and nucleic acid based sensing.[7,8] The past few years have also witnessed some advances in this topic through the use of nonbiological, synthetic catalysts based on metal-containing substances such as palladium salts, palladium nanoparticles, and organoruthenium complexes.[2–6] Most notably, Bradley and co-workers reached reasonably high catalytic activities of up to 30 turnovers in an uncaging reaction with palladium-modified resins—although the efficiency was only determined under biologically relevant conditions and not within a living system.[5a] As a consequence of the stringent requirements set by the cellular environment, it is, despite these first accomplishments, still highly challenging to achieve multiple substrate turnovers with an artificial catalyst inside a biological system.

Herein, we present our progress towards organometallic ruthenium complexes for the catalytic uncaging of allyloxycarbonyl-protected amines under biologically relevant conditions and within living mammalian cells (Scheme 1). The high turnover numbers of the new catalyst/substrate pairs enabled us to catalytically activate an anticancer prodrug inside HeLa cells, and hence switch the cellular viability from healthy to apoptotic.



Scheme 1. This study and previous work (see Ref. [4a]) on the catalytic cleavage of *O*-allyl carbamates with organoruthenium complexes under biologically relevant conditions. TON = turnover number.

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Almost a decade ago we reported that the organometallic half-sandwich complex [Cp*Ru(cod)Cl] (**Ru1**; Cp* = η^5-pentamethylcyclopentadienyl, cod = η^4-1,5-cyclooctadiene) can catalyze the conversion of bioorthogonal *O*-allyl carbamates into their respective amines under biologically relevant conditions (presence of water, air, and thiols) and inside living HeLa cells, albeit with such a low catalytic activity that it was challenging to develop meaningful applications (Scheme 1).[4a,9] To identify more sophisticated organometallic catalyst/substrate pairs, we started our study by evaluating a set of ruthenium half-sandwich complexes that are known to be efficient Tsuji–Trost catalysts.[10] We examined these complexes in a deallylation reaction with *N*-(allyloxycarbonyl)-aminocoumarin **1** in water, under air, and in the presence of glutathione (GSH; 5 mM; Figure 1).[11] The new water-soluble caged fluorophore **1** enabled us to keep the amount of undesired organic co-solvents to a minimum, thereby staying as close to biologically relevant conditions as possible. To mimic typical compound concentrations used in cellular assays, we furthermore chose a substrate concentration of 500 μM, which is several orders of magnitude lower than the concentrations found in typical reactions for chemical synthesis.[12] The results are shown in Figure 1 and demonstrate that almost no catalytic activity was found for the canonical acetonitrile complexes [CpRu(MeCN)$_3$]PF$_6$ (Cp = η^5-cyclopentadienyl) and [Cp*Ru(MeCN)$_3$]PF$_6$ and only a low activity for the 2,2′-bipyridine (bpy) complex [Cp*Ru(bpy)(MeCN)]PF$_6$ under these challenging reaction conditions. Even though our previously reported catalyst **Ru1** displayed a significant initial reaction rate in combination with an excess of thiophenol (PhSH), it quickly became deactivated (within less than 30 min). However, to our surprise, the complex [CpRu(QA)(η^3-allyl)]PF$_6$ (**Ru2**; QA = 2-quinolinecarboxylate) provided full conversion at a catalyst loading of merely 5 mol% within 3 h, thus combining the high reactivity of our previous catalyst with long-standing activity.

The quinoline complex **Ru2** was first described by Kitamura and co-workers and is commonly used in (de)-allylation reactions with various nucleophiles.[13] To analyze the structure–activity relationship of **Ru2**, we evaluated the three homologous Cp* and 2-pyridinecarboxylato (PA) derivatives which were reported to catalyze *S*-allylation reactions.[14] These catalysts ([CpRu(PA)(η^3-allyl)]PF$_6$, [Cp*Ru(QA)(η^3-allyl)]PF$_6$, and [Cp*Ru(PA)(η^3-allyl)]PF$_6$) were found to exceed most complexes tested so far in our laboratory, although could not reach the high catalytic activity of **Ru2** (Table 1). We believe that the reduced reactivity of the Cp* complexes is due to steric hindrance by the five methyl groups[15] and that the fast deactivation rate of the pyridine complexes is a consequence of the electronic properties of the bidentate ligand—particularly its π-accepting ability.[16] We therefore hypothesized that an even higher catalytic activity for the quinoline complexes could be attained once the π backbonding of the bidentate ligand was reduced. Indeed, when we introduced a π-donating methoxy (**Ru3**) or dimethylamino (**Ru4**) group into the quinoline moiety, we observed a significant boost in activity, as exemplified by the turnover numbers reached (TON; **Ru2** = 90, **Ru3** = 150, and **Ru4** = 270; see the Supporting Information for more details). Even with only 1 mol% catalyst loading, the dimethylamine catalyst **Ru4** achieved full deprotection of the caged fluorophore **1** within about 4 h under biologically relevant conditions (Table 1).

Table 1: Catalytic deprotection of **1** with organoruthenium complexes derived from **Ru2** under biologically relevant conditions.[a]

Entry		Complex	Cat. [equiv]	Yield [%][b] 1 h	4 h	24 h
1	**Ru2**	[CpRu(QA)(allyl)]PF$_6$	0.05	74	>99	>99
2		[CpRu(PA)(allyl)]PF$_6$	0.05	43	80	85
3		[Cp*Ru(QA)(allyl)]PF$_6$	0.05	10	38	84
4		[Cp*Ru(PA)(allyl)]PF$_6$	0.05	3	10	52
5	**Ru2**	[CpRu(QA)(allyl)]PF$_6$	0.01	16	47	65
6	**Ru3**	[CpRu(QA-OMe)(allyl)]PF$_6$	0.01	32	79	93
7	**Ru4**	[CpRu(QA-NMe$_2$)(allyl)]PF$_6$	0.01	29	91	>99

[a] Reaction conditions: see Figure 1. [b] Yields were determined by the fluorescence intensity (λ_{ex} = 395 nm, λ_{em} = 460 nm). The mean values of three independent measurements are shown. QA-OMe = 4-methoxy-2-quinolinecarboxylate, QA-NMe$_2$ = 4-(*N*,*N*-dimethylamino)-2-quinolinecarboxylate.

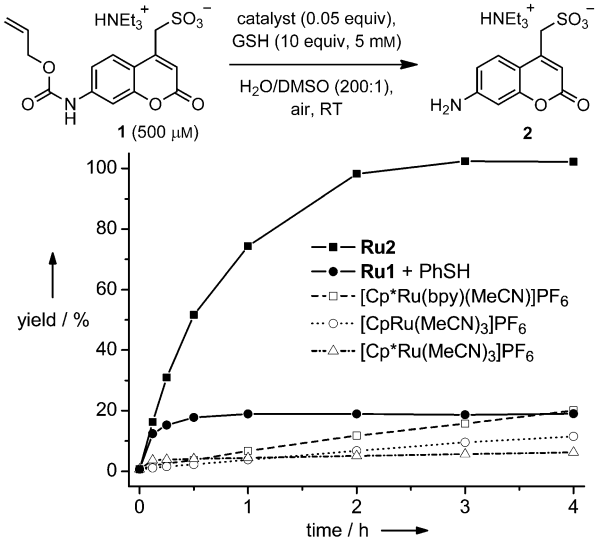

Figure 1. Catalytic deprotection of **1** with organoruthenium complexes under biologically relevant conditions. Reaction conditions: **1** (1.0 equiv, 500 μM), catalyst (0.05 equiv, 25 μM), and glutathione (GSH; 10 equiv, 5 mM) in water/DMSO (200:1) at room temperature and in the presence of air. For **Ru1**, thiophenol (PhSH; 5.0 equiv, 2.5 mM) was added to the reaction mixture. Yields were determined by the fluorescence intensity (λ_{ex} = 395 nm, λ_{em} = 460 nm). The mean values of three independent measurements are shown.

Interestingly Kitamura and co-workers found an opposite reactivity trend for analogous quinoline complexes in the allylation of alcohols and deallylation of allyl ethers.[16] They observed that a reduced π backbonding of the bidentate ligand results in a diminished catalytic activity and concluded that this effect can be attributed to a lower electrophilicity of the η^3-allyl ligand. This means that in the presence of weak nucleophiles, such as alcohols, the allylation step is rate-determining. However, our own mechanistic investigations on

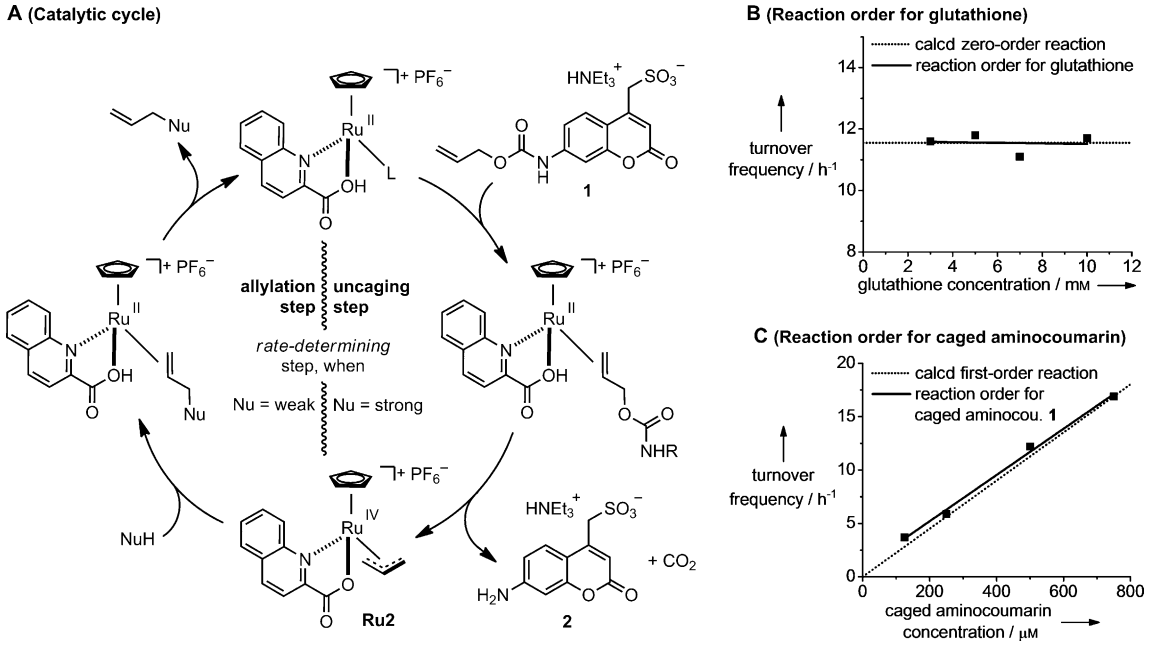

Figure 2. A) Catalytic cycle for the deprotection of **1** with **Ru2** under biologically relevant conditions. Nu = nucleophile (e.g. glutathione), L = undefined ligand (e.g. water). B) Reaction order with respect to glutathione in the uncaging reaction of **1**. Reaction conditions: **1** (1.0 equiv, 500 μM), **Ru2** (10^{-3} equiv, 0.5 μM), and glutathione (6–20 equiv, 3–10 mM) in water/DMSO (200:1) at room temperature and in the presence of air. Turnover frequency was determined after 30 min by the fluorescence intensity ($\lambda_{ex} = 395$ nm, $\lambda_{em} = 460$ nm). The mean values of three independent measurements, linear regression curve, and calculated curve for zero-order reaction are shown. C) Reaction order with respect to **1** for the uncaging reaction of **1**. Reaction conditions: **1** (125–750 μM), **Ru2** (0.5 μM), and glutathione (5 mM) in water/DMSO (200:1) at room temperature and in the presence of air. The turnover frequency was determined after 30 min by the fluorescence intensity. The mean values of three independent measurements, linear regression curve, and calculated curve for a first-order reaction are shown.

Ru2 suggest that in the presence of excess strong thiol nucleophiles (e.g. glutathione) the rate-determining step shifts from the allylation step towards the uncaging step (Figure 2). Further investigations revealed, moreover, that the reaction rate is not influenced by the glutathione concentration under biologically relevant conditions (zero-order rate). These results let us assume that the millimolar thiol concentrations inside living cells do not diminish the catalytic activity of the ruthenium catalysts, but instead enable a fast reaction to occur, since the allylation step is no longer limiting the reaction rate.

The high catalytic activity of the quinoline complexes **Ru2**, **Ru3**, and **Ru4** under biologically relevant conditions encouraged us to evaluate the new catalyst/substrate pairs under cellular conditions (Figure 3). For this, HeLa cells were grown on imaging dishes and incubated with bis[*N*-(allyloxycarbonyl)]rhodamine 110 (**3**; 100 μM) for 30 min. After washing the cells twice with phosphate-buffered saline (PBS) to remove extracellular caged fluorophore, fresh medium and catalyst (20 μM) were added. Uptake of the catalyst should now result in the fluorescence intensity only increasing inside the cells where the caged probe is located. Indeed, the intensity of green fluorescence increased remarkably (**Ru2** = 70-fold, **Ru3** = 90-fold, and **Ru4** = 130-fold) within the cytoplasm over 10 min, as evident by observing changes in the fluorescence by live-cell imaging with a confocal fluorescence microscope. In control experiments, we verified that no fluorescence evolves in the absence of the catalyst and only moderate fluorescence (8-fold) when the significantly less efficient **Ru1** complex is used in combination with toxic thiophenol. These experiments demonstrate that the quinoline complexes **Ru2**, **Ru3**, and **Ru4** are capable of passing readily through the cell membrane and that they are able, moreover, to efficiently uncage *O*-allyl carbamates within living cells. In addition, it is noteworthy that we observed the same reactivity trend (**Ru2** < **Ru3** < **Ru4**) in living cells as already measured under biologically relevant conditions (Table 1).

Finally, to demonstrate the potential of bioorthogonal organometallic catalyst/substrate pairs for future applications we tested our efficient **Ru4**/allyl carbamate system for use in cancer therapy. A great aim in modern cancer therapy is the spacial and temporal activation of prodrugs inside cancerous tissue so as to avoid damaging surrounding healthy tissue during treatment.[17] We believe that the *catalytic* activation of chemotherapeutic agents inside tumors is possible and might be a new approach for future treatments. The first example in this new field of medicinal chemistry was recently reported by Bradley, Unciti-Broceta, and co-workers.[5e] They showed that catalytically active palladium-functionalized polystyrene microspheres are able to activate propargyl-protected 5-fluorouracil in the extracellular space. Upon uncaging and subsequent uptake of the potent anticancer drug 5-fluorouracil, a loss of cellular survival was observed for different cell lines within 5 days. We believe that, in comparison to the extracellular catalysis, the activation of a prodrug within the cellular cytoplasm might have an enhanced therapeutic effect along with reduced adverse drug reactions. For this reason,

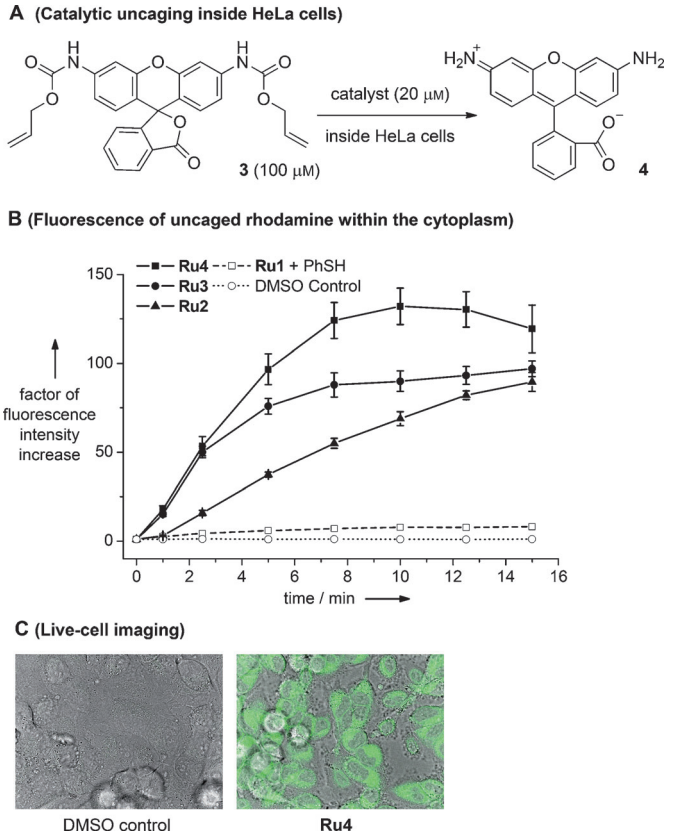

Figure 3. A) Catalytic deprotection of **3** with organoruthenium complexes inside HeLa cells. B) Time-dependent increase in the fluorescence intensity within the cellular cytoplasm determined by fluorescence microscopy ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 500$–555 nm). Reaction conditions: HeLa cells were grown on imaging dishes (35 mm/12 mm/180 μm; 350000 cells/dish), incubated with **3** (100 μM) for 30 min, washed with PBS buffer, and incubated in fresh medium with catalyst (20 μM) at 37°C. For **Ru1**, thiophenol (250 μM) was also added. For the control reaction, 1% DMSO was added instead of the catalyst. Intensity increase is given as a mean value of eight cells. C) Superimposed bright-field and confocal fluorescence images after 10 min of incubation with 1% DMSO (control reaction) or 20 μM **Ru4**.

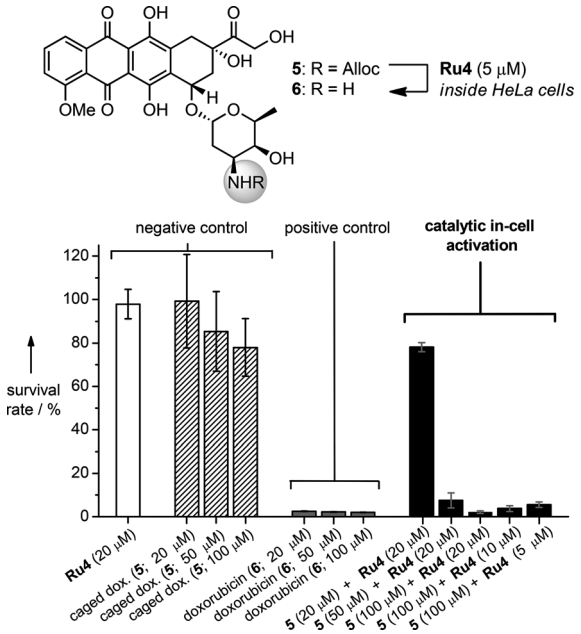

Figure 4. Catalytic deprotection of **5** (Alloc = allyloxycarbonyl) with **Ru4** inside HeLa cells. Reaction conditions: HeLa cells were grown in 96-well plates (9000 cells/well); white bar: negative control; cells were incubated with 1% DMSO for 3 h, washed with PBS buffer, and incubated in fresh medium with **Ru4** (20 μM) for 24 h; hatched bar: negative control; cells were incubated with **5** (20–100 μM) for 3 h, washed with PBS buffer, and incubated in fresh medium with 1% DMSO for 24 h; gray bar: positive control; cells were incubated with **6** (20–100 μM) for 3 h, washed with PBS buffer, and incubated in fresh medium with 1% DMSO for 24 h; black bar: catalytic in-cell activation; cells were incubated with **5** (20–100 μM) for 3 h, washed with PBS buffer, and incubated in fresh medium with **Ru4** (5–20 μM) for 24 h. Survival rates were determined by the MTT method. The mean values of two independent experiments (9 wells each) are shown. Caged dox. = protected doxorubicin.

and since anthracyclines are reported to induce fast apoptosis at low concentrations, we developed an in-cell uncaging strategy with the frequently used anticancer agent doxorubicin.[18] The cytotoxic effect of doxorubicin can mainly be attributed to DNA binding and successive strand damage.[19] To disable the toxicity of doxorubicin, we lowered the DNA affinity by masking the positive charge of the primary amine with the bioorthogonal allyl carbamate protecting group. As expected, the prodrug *N*-(allyloxycarbonyl)doxorubicin (**5**) showed no toxicity, while the free drug doxorubicin (**6**) induced apoptosis within hours after incubation (Figure 4). As long as the HeLa cells were incubated with the prodrug **5** at 20 μM, washed with PBS buffer, and incubated with **Ru4** (20 μM) in fresh medium, we detected only a small decrease in cellular survival after 24 h. However, when we increased the concentration of the nontoxic prodrug to 50 μM and 100 μM, we observed an efficient reduction in viable cells in a concentration-dependent fashion [$(7.5 \pm 3.5)\%$ and $(2.1 \pm 0.6\%)$]. Since the catalyst and the prodrug do not show any toxicity at these concentrations, we conclude that this dramatic change in viability is due to the efficient uncaging of the prodrug **5** inside the cytoplasm (for the uncaging of **5** under biologically relevant conditions, see the Supporting Information). At the higher concentration of the caged doxorubicin **5** (100 μM) a significantly reduced catalyst loading of just 10 μM and 5 μM **Ru4** could even be employed for the efficient induction of cellular apoptosis, which led to cellular survivals of $(3.7 \pm 1.3)\%$ and $(5.5 \pm 1.3)\%$, respectively. In a control experiment, we tried to activate the caged doxorubicin **5** (50 μM) with the less efficient catalyst **Ru1** (20 μM), but, as expected, we could not observe any change in viability because of the lower catalytic activity (see the Supporting Information). In contrast to the kinetic experiments described above, the thiophenol additive could this time not be used to boost the catalytic efficiency of catalyst **Ru1** because of its intrinsic toxicity. These cellular experiments show clearly that a high catalyst activity as well as stability will be beneficial for the activation of organometallic prodrugs in future medical applications.

In conclusion, we have presented new bioorthogonal organometallic complexes for catalytic uncaging reactions of allyloxycarbonyl-protected amines. The reported catalysts

exhibit unprecedented high turnover numbers of up to 270 cycles under biologically relevant conditions and efficient activity within mammalian cells. We showed that the ligand design has a major influence on the stability and reactivity of the catalysts under biologically relevant conditions as well as within cellular cytoplasm. We further demonstrated that the stringent requirements of a cellular environment, such as millimolar concentrations of thiols, are not necessarily detrimental to a catalyst, but can actually be helpful for a fast reaction. In addition, we performed a catalytic activation of caged doxorubicin within the cellular cytoplasm which changed the viability of HeLa cells in an effective on/off mode from healthy to apoptotic. Even though this application is just a first example and not yet fully developed for cancer treatment, we nonetheless expect great results from future bioorthogonal organometallic catalyst/substrate pairs with significant value for chemical biology and medicinal chemistry. Noteworthy advantages of the presented catalysts is the fact that they can passively diffuse into cells and can be combined with other biomedical technologies such as targeted drug-delivery systems[20] to yield merged systems that, in the end, are specific for certain tissues or cytoplasmic structures.

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