An artificial metalloenzyme for carbene transfer based on a biotinylated dirhodium anchored within streptavidin

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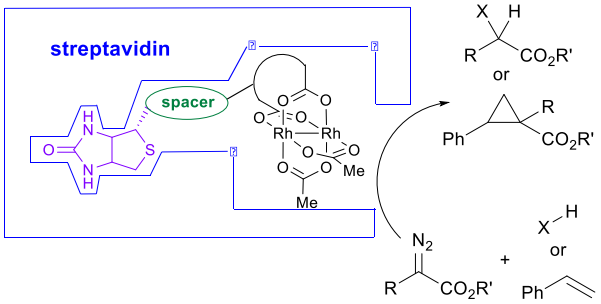
We report on artificial metalloenzymes that incorporate a biotinylated dirhodium core embedded within engineered streptavidin (Sav hereafter) variants. The resulting biohybrid catalyzes the carbene insertion in C–H bonds and olefins. Chemical- and genetic optimization allows to modulate the catalytic activity of the artificial metalloenzymes that are shown to be active in the periplasm of *E. coli* (up to 20 turnovers).

Dirhodium(II) tetracarboxylate complexes have been shown to be exceptionally active catalysts for carbene-transfer reactions including cyclopropanation[1–3] and X-H insertion (X= C, N, O, S, Si etc)[4–8]. Thanks to their robustness, remarkable activity and selectivity under physiological conditions, they have also found applications as a versatile tool in chemical biology. For this purpose, dirhodium tetracarboxylate moieties have been linked to biomacromolecules including peptides, proteins and oligonucleotides. The Ball group developed dirhodium metallopeptides for protein modification and intracellular imaging.[9,10] Gillingham and coworkers have reported dirhodium complexes for DNA modification[11], aqueous catalysis and metal uptake within tumor cells[12]. In the context of artificial metalloenzymes (ArMs), the Lewis group covalently linked a dirhodium tetracarboxylate bearing a terminal alkyne to a genetically-engineered prolyl oligopeptidase equipped with an azidophenylalanine. The resulting ArM displayed excellent catalytic properties for intermolecular cyclopropanation.[13] Relying on directed evolution (either random or targeted), both the activity and the enantioselectivity could be significantly improved.[14] The evolved dirhodium cyclopropanase was further shown to catalyze other carbene-transfer reactions.



Scheme 1 Artificial metalloenzymes for carbene transfer based on a dirhodium tetracarboxylate moiety. The catalytic properties of the ArM can be chemo-genetically optimized: variation of the spacer (green) between the biotin anchor and the dicarboxylate moiety can be combined with the introduction of point mutations on the streptavidin scaffold (blue stars).

Pioneered by Wilson and Whitesides in 1978, artificial metalloenzymes based on the biotin-(strept)avidin technology, have been implemented for a large variety of reactions including: (transfer)-hydrogenation[15,16], cross-coupling[17], metathesis etc.[18–20] Since then, many groups have reported on the creation of various ArMs relying on alternative anchoring strategies with promising catalytic properties.[21–28] Building upon our experience with ArMs based on the biotin-streptavidin technology relying on precious metal cofactors,[29,30] we present herein our efforts to anchor a bulky biotinylated dirhodium moiety within engineered streptavidin to catalyze carbene transfer reactions, Scheme 1.

Initially, we designed four bis-chelating dirhodium cofactors **1**-**4** (Scheme 2). Substitution of either two acetate- or trifluoracetate ligands from $Rh_2(OAc)_4$ and (*cis*)-$Rh_2(OAc)_2(OCOCF_3)_2$[31] respectively with the *m*-substituted dicarboxylate-bearing ligands **1b**, **2c**, **3c**, **4b** yielded the dirhodium intermediates **1c**, **2d**, **3d**, **4c** respectively (see ESI for experimental details). Biotinylation using biotin derivatives **7a**

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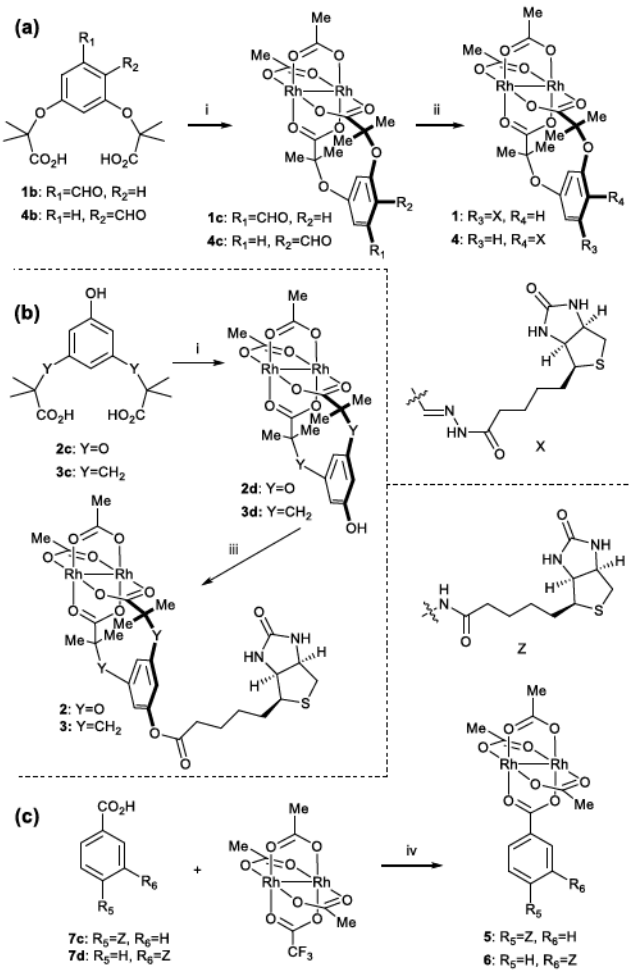
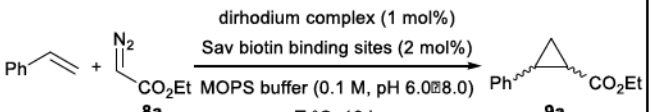


Scheme 2 Biotinylated dirhodium tetracarboxylate complexes tested in this study: (a) catalyst **1** and **4**; (b) catalyst **2** and **3**; (c) catalyst **5** and **6**. Reaction conditions: i) $Rh_2(OAc)_4$, *N*, *N*-dimethylaniline, 140 °C, 3 h or (*cis*)-$Rh_2(OAc)_2(OCOCF_3)_2$, K_2CO_3, THF, 50 °C, 3 h; ii) (+)-biotin hydrazide **7a**, TFA, DMSO, rt, 3 h; iii) biotin pentafluoro phenyl ester **7b**, NaH, DMSO, rt, 3 h; iv) *N*, *N*-Diisopropylethylamine, DMSO, 50 °C, 2 h.

or **7b**, through either a hydrazine coupling or an esterification, afforded the target biotinylated dirhodium cofactors **1**-**4**. In light of the remarkable inertness of the $Rh_2(^2O_2CR)_4$ moiety, we also prepared the dirhodium complexes **5** and **6**. For this purpose, $Rh_2(OAc)_3(OCOCF_3)$[31] was treated with biotinylated monocarboxylic acid **7c** or **7d** to afford the corresponding complexes **5** and **6** respectively. All biotinylated dirhodium complexes were purified by reversed-phase preparative HPLC and fully characterised (See SI).

In the absence of Sav, the dirhodium complexes **1**-**6** (25 °C, pH 7.0, MOPS buffer (0.1 M)) outperform (i.e. higher turnover number, TON) $Rh_2(OAc)_4$ for the cyclopropanation of styrene (Table 1, entries 1-7). Incorporation of the dirhodium cofactors **1**-**6** into wild-type streptavidin (Sav WT) leads to an erosion in activity (Table 1, entries 9-14). Cofactor **2** outperforms all other cofactors: **2** · Sav WT affords 54 TON, compared to 79 TON in the absence of Sav. The biotinylated monodentate dirhodium complexes **5** and **6** lead to significantly lower TONs

Table 1 Selected results for the ArMs-catalysed cyclopropanation of styrene with ethyl diazoacetate **8a**.[a]



Entry	Catalyst	Sav	Tem (°C), pH	TON[b]	*trans*/*cis*
1	$Rh_2(OAc)_4$		25, 7.0	2 ± 0	1.5/1
2	**1**		25, 7.0	68 ± 1	1.4/1
3	**2**		25, 7.0	79 ± 0	1.3/1
4	**3**		25, 7.0	79 ± 1	1.4/1
5	**4**		25, 7.0	52 ± 1	1.3/1
6	**5**		25, 7.0	60 ± 1	1.4/1
7	**6**		25, 7.0	46 ± 1	1.4/1
8	$Rh_2(OAc)_4$	WT	25, 7.0	2 ± 0	1.5/1
9	**1**	WT	25, 7.0	34 ± 3	1.3/1
10	**2**	WT	25, 7.0	54 ± 7	1.4/1
11	**3**	WT	25, 7.0	38 ± 2	1.4/1
12	**4**	WT	25, 7.0	33 ± 1	1.3/1
13	**5**	WT	25, 7.0	3 ± 0	1.1/1
14	**6**	WT	25, 7.0	1 ± 0	1.3/1
15	**2**	WT	5, 7.0	4 ± 1	1.2/1
16	**2**	WT	55, 7.0	0	–
17	**2**	WT	25, 4.0[c]	2 ± 0	1.3/1
18	**2**	WT	25, 5.0[d]	29 ± 4	1.4/1
19	**2**	WT	25, 6.0	50 ± 6	1.4/1
20	**2**	WT	25, 8.0	12 ± 4	1.4/1

[a] Reaction conditions: [styrene] = 10 mM, [**8a**] = 30 mM, [catalyst] = 100 µM, [Sav biotin binding sites] = 200 µM, V_{tot} = 400 µL (5% DMSO), 25 °C for 16 hours. [b] TON = turnover number determined by GC using 1, 3, 5-trimethoxybenzene as internal standard. The ± values represent standard deviations resulting from independent reactions perfomed in duplicate. [c] acetate buffer (0.1 M, pH 4.0). [d] acetate buffer (0.1 M, pH 5.0).

when embedded with Sav. We hypothesize that this may be due to the loss of the biotinylated ligand accompanied by decomposition of the dirhodium moiety. Increasing or reducing the temperature leads to an erosion in TON for **2** · Sav WT (Table 1, entries 15, 16). Screening at various pH highlights that the dirhodium cofactor **2** performs best at neutral pH (Table 1, entries 17-20). Based on this initial screen, cofactor **2** was selected for all further investigations.

For genetic optimization purposes, we screened purified Sav mutants for both cyclopropanation and C-H insertion reactions in the presence of cofactor **2**. The results are summarized in Figure 1a using ethyl diazoacetate **8a** and Figure 1b using donor-acceptor ethyl diazo(phenyl)acetate **8b** respectively.

In the presence of the more reactive ethyl diazoacetate **8a,** none of the screened mutants outperformed the wild-type artificial cyclopropanase **2** · Sav WT. Introduction of Lewis-basic amino acids in the proximity of the dirhodium moiety (i.e. S112C, S112H, K121C, K121D) yielded comparable TONs to **2** · Sav WT. Introduction of two Lewis-basic aminoacid residues (ie. S112H-K121H; S112C-K121H) lead to a significant erosion in TONs. Similar results were obtained upon introducing hydrophobic residues within the biotin-binding vestibule: none of the mutants screened led to an increase in catalytic performance (ie. S112A, S112W, K121A, K121F, K121W etc., Figure 1a). Screening in the presence of the bulky donor-

acceptor diazo(phenyl)acetate **8b** afforded significantly lower TONs. In this case however, some mutants bearing Lewis-basic side chains performed slightly better than **2** · Sav WT: S112D, S112K, S112H, K121E, Figure 1b. Unfortunately, incorporation of the biotinylated dirhodium cofactor **2** within Sav had no influence on either the diastereoselectivity (i.e. *trans/cis* ratio) or the enantioselectivity (i.e. racemic product) of the cyclopropanation.

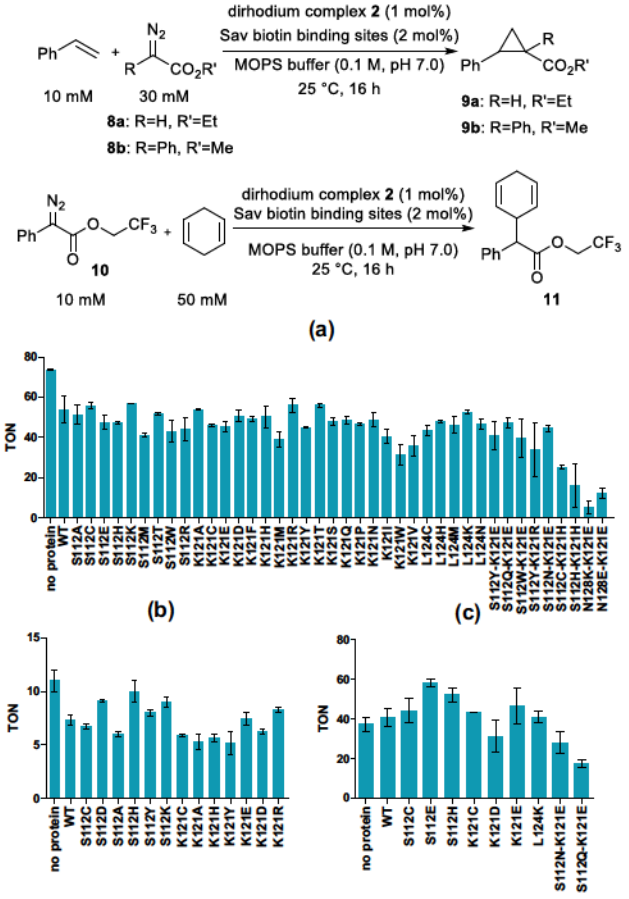


Fig. 1 Experimental conditions and selected results for the ArMs catalysed cyclopropanation of styrene with ethyldiazoacetate **8a** (a) and diazo(phenyl)acetate **8b** (b), and C-H insertion of diazo **10** with 1,4-cyclohexadiene (c). The error bars represent standard deviations resulting from independent reactions performed in duplicate.

Next, we investigated the C-H insertion of trifluoroethyl (phenyl)diazoacetate **10** with 1, 4-cyclohexadiene catalysed by **2** · Sav and mutants thereof. The reaction afforded exclusively the allylic insertion product **11**, with no double insertion or cyclopropanation by-products detected. A selection of Sav mutants were screened. Gratifyingly, **2** · Sav S112E and K121E outperformed both the free cofactor **2** and **2** · Sav WT. No enantioselectivity could be detected however.

Unfortunately, all attempts to obtain crystals suitable for X-ray diffraction of **2** · Sav were unsuccessful. Thus, to gain structural insight into the localisation of biotinylated dirhodium complex within Sav WT, a docking simulation for the hydrated dirhodium complex **2**·$(H_2O)_2$ within Sav WT was performed using the GOLD software suite, Figure 2.[32] The minimized docked structure suggests that the dirhodium moiety protrudes out of the biotin-binding vestibule: The shortest C - Rh distances are: 6.1 Å, 6.0 Å, 7.4 Å for the closest lying amino acids S112, K121 and L124 respectively, Figure 2. This may explain why the genetic optimization has such a modest effect on the performance of the ArM.

It has been reported that dirhodium tetracarboxylate moieties tolerate cellular components.[33] We thus secreted the Sav to the periplasm and screened the artificial cyclopropanase in the

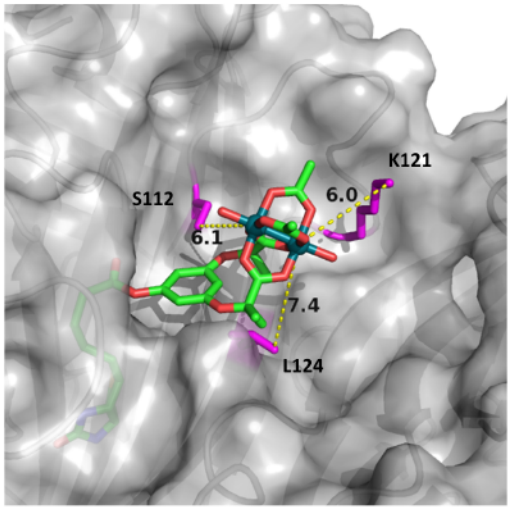


Fig. 2 Structure resulting from a docking simulation of dirhodium complex **2**·$(H_2O)_2$ · Sav WT. The protein is displayed as light grey surface and the biotinylated cofactor **2** is displayed as color-coded sticks. The closest lying amino acids are displayed as magenta sticks. The closest contacts between the cofactor and S112, K121 and L124 are highlighted with yellow dotted lines.

presence of whole *E. coli* cells. Figure 3 outlines the protocol implemented for the periplasmic screening.[34] The TOP10(DE3)_pET30 strain was used to express and secrete Sav into the periplasm. The following isoforms were tested: Sav WT, S112C, S112D, K121C and K121M. After harvesting, the cell pellets were incubated with a MOPS buffer containing 50 µM dirhodium cofactor **2** for 30 min on ice. The unbound cofactor **2** was washed away and the catalysis buffer containing the substrate was added to the cell pellet. Gratifyingly, the artificial cyclopropanase **2** · Sav WT outperformed the free cofactor **2**. Introduction of a cysteine residue, either at position S112C or K121C leads to improved activities. ICP-MS analysis revealed significant Rh-accumulation within *E. coli* harbouring periplasmic Sav (Sav^{peri}): *E. coli* not secreting Sav (empty *E. coli*) contained 3.2 nmol Rh (corresponding to 6% uptake from the 50 nmol Rh-added to the supernatant). For *E. coli* harbouring Sav^{peri}, the Rh-amounts determined varied between 5.6 – 8.9 nmol (i.e. 11% – 18% uptake) depending on the mutant. Accordingly, the TONs are 10 for the empty *E. coli*, 9 for Sav^{peri} WT, 17 for Sav^{peri} S112C and 20 for Sav^{peri} K121C respectively (See SI).

In summary, we have developed an artificial carbenoid transferase based on the biotin-streptavidin technology. The

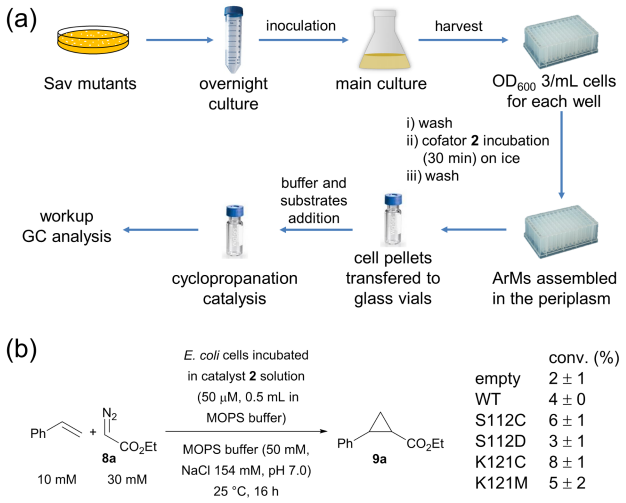


Fig. 3 An *in cellulo* artificial cyclopropanase workflow (a), reaction conditions and screening results (b) for the periplasmic screening. The ± values represent standard deviations resulting from independent reactions performed in duplicate.

resulting ArMs catalyse both intermolecular cyclopropanation and C-H insertion. Importantly, we have demonstrated that the dirhodium ArM maintains its activity in the periplasm of *E. coli* cells. The performance of the ArM can be optimized by genetic means, outperforming both the free cofactor and the **2** · Sav WT. Although the TONs reported *in vivo* remain modest (20 TONs for **2** · Sav^{peri} K121C), these compare favorably to the catalytic systems typically used in chemical biology, whereby the organometallic "catalyst" is often used in (super) stoichiometric quantities.[35,36] These findings pave the way for high-throughput screening to further optimize the activity and the selectivity of such artificial carbenoid-transferases based on the biotin-streptavidin technology. In this context, we have recently reported on chimeric Sav that incorporate extended loops around the biotin-binding vestibule.[37] We hypothesize that these chimeras may offer a better-defined environment around the bulky dirhodium cofactor **2**, thus offering a means to influence the selectivity of the reactions.

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Conflicts of interest

The authors declare no conflicts of interest.

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